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(54) Title: ZONA PELLUCIDA RELATED OLIGOSACCHARIDES

(57) Abstract

In this invention oligosaccharides obtainable by degradation of zona pellucida glycoproteins are presented, specifically O-linked oligosaccharides, which can be used for immunecontraceptive vaccines. Also copolymers and conjugates comprising the oligosaccharides are part of the invention. Furthermore the invention comprises antibodies to the oligosaccharides and pharmaceutical compositions with these oligosaccharides or antibodies.

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Zona Pellucida related oligosaccharides

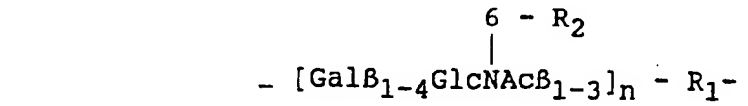
This invention relates to oligosaccharides obtainable by degradation of zona pellucida (ZP) glyco proteins, more specifically O-linked oligosaccharides from porcine ZP, conjugates with these oligosaccharides, antibodies against these oligosaccharides, vaccines, pharmaceutical compositions and diagnostics with these oligosaccharides.

The first interaction between mammalian gametes during the process of fertilization is mediated by the binding of sperm cells to (a) species specific ligand(s) on the oocyte zona pellucida (ZP). The ZP is an extracellular matrix that surrounds the female gamete and that comprises at least three glycoproteins, designated ZP1, ZP2 and ZP3. A number of studies have indicated that the initial interaction between sperm and oocyte is mediated by a protein-carbohydrate recognition system, involving specific sperm proteins and carbohydrate chains (oligosaccharides) of ZP3 (reviewed in P.M. Wassarman, Development 108, 1-17, 1990).
Prevention of the interaction between sperm cell and oocyte would introduce a non-steroidal method for contraception. Such method for contraception can be achieved by blocking of the sperm receptor ligands on the zona pelludica with antibodies directed against these ligands. This method of contraception is generally referred to as immunocontraception. The antibodies which prevent said interaction between the sperm cell and oocyte are referred to as contraceptive antibodies. Antibodies displaying such contraceptive activity can be administered by subcutaneous injection (passive immunization) or can be produced by the body itself upon vaccination with (part of) the ligand molecule (active immunization).

Methods for immunocontraception have been described. In JP 63/150299 and WO 89/03398 the use of zona pellucida glycoproteins for contraceptive vaccination has been described. These vaccines comprise either the polypeptide backbone or the whole glycoprotein. Since the zona pellucida proteins are macromolecules with high molecular weights, these vaccines give rise to a great variety of antibodies. These antibodies are directed to carbohydrate moieties of the glycoprotein, to epitopes on the polypeptide backbone as well as to certain conformational structures of the whole glycoprotein. As not the whole glycoprotein is involved in the sperm-oocyte interaction but only specific parts (the so called ligands), only those antibodies which are directed to those ligands will display contraceptive activity. Immunocontraception based on the whole glycoprotein or the polypeptide backbone thereof is therefore not a very efficient approach. To circumvent the arisal of redundant antibodies against the zona pellucida protein, contraceptive vaccines are needed which will specifically direct the elicited immuneresponse towards one of the ligands of the sperm receptor on the oocyte.

It is an object of the invention to provide a new compound which can be used for contraceptive vaccination, said compound specifically giving rise to antibodies with contraceptive activity. It is a further aspect of the invention to provide antibodies raised against this new compound. Another aspect of the invention is to provide new pharmaceutical compositions and contraceptive vaccines.

The present invention provides such a compound. It was found that the isolated O-linked oligosaccharides from zona pellucida proteins are suitable ligands for use in a vaccine because the antibodies raised to these oligosaccharides have contraceptive activity.
These oligosaccharides are new and comprise the following structure:



with n = 1-10

R_1= $Gal\beta_{1-3}GalNAc$, and

R_2= OH or OSO_3^- or $\alpha_{2-6}NeuAc$

or pharmaceutically acceptable salts thereof.

The N-Acetyllactosamine units, especially from second repeat onward, preferably contain a sulphate group linked at the C-6 position of GlcNAc. In the terminal non-reducing position of the oligosaccharides an α_{2-3} linked sialic acid residue (NeuAc or NeuGc) may be present. Preferably, in order to inhibit the sperm-oocyte interaction, the oligosaccharides contain the tetrasaccharide core in combination with one or more extra 6-O-sulfated N-acetyllactosamine units.

The oligosaccharides according to the invention are obtainable from the O-linked carbohydrate chains of zona pellucida glycoproteins by degradation and working up procedures. Preferably the oligosaccharides are obtained from porcine O-linked zona pellucida carbohydrate chains.

Recently the N-linked carbohydrate chains have been elucidated (Noguchi, S. and Nakano, M., Eur. J. Biochem. 209, 883-894, 1992) and it was stated that the neutral N-linked oligosaccharides retain the receptor activity

whereas the acidic N-linked oligosaccharides do not possess any receptor activity. However no suggestion was made towards the capacity of the O-linked oligosaccharides to raise antibodies with contraceptive activity. Nor do they suggest the use of said oligosaccharides in a vaccine for contraceptive acctivity. EP-A-298064 discloses the use of specific carbohydrate structures and antibodies raised against these structures. No oligosaccharides according to the presenty invention are disclosed; and the antibodies against those carbohydrate structures do not prevent the interaction between sperm cell and oocyte, but inhibit the uterine implantation of the embryo resulting from conception instead.

Thus, the oligosaccharides according to the invention can be used in a vaccine for contraceptive treatment. Recently it has been shown that in mice sperm cells bind to the O-linked oligosaccharides of the ZP3 glycoprotein (Wasserman et al., Biol. Repr. 46:186-191, 1992). The advantage of a vaccine comprising an oligosaccharide according to the invention is that an immuneresponse solely directed to a ligand participating in the sperm-oocyte interaction is elicited.

For the use of the oligosaccharides according to the invention in a vaccine it is preferable to enhance the immunogenic effect of the epitope-bearing oligosaccharides by crosslinking them to immunogenic carriers or to copolymerize these oligosaccharides.

Preferably the oligosaccharides to be used in a contraceptive vaccine have the following structure:

$$R_3 - [Gal\beta_{1-4}GlcNAc\beta_{1-3}]_n - R_1$$
$$\qquad\qquad\qquad\qquad\quad |$$
$$\qquad\qquad\qquad\qquad\quad 6 - R_2$$

with n = 1-10

R_1= $Gal\beta_{1-3}GalNAc_{\alpha/\beta 1}-R_4$ or $Gal\beta_{1-3}GalNAc$-ol

R_2= OH or OSO_3 or $\alpha_{2-6}NeuAc$

R_3= H, $6SO_4$, $4SO_4$, $3SO_4$, $\alpha_{2-3}NeuAc$, $\alpha_{2-3}NeuGc$, $\alpha_{2-6}NeuAc$, $\alpha_{2-6}NeuGc$, GlcNAc, $GlcNAc-_6SO_4$,

R_4 = H, or a group for polymerization or for conjugation to the immunogenic carrier.

For conjugation to a carrier molecule the oligosaccharides are equipped with a spacer molecule at the anomeric carbon atom of the reducing end monosaccharide. The linkage can be α or ß. Suitable spacers are well known in the art. Examples are succinimidyl, thiol, amino, carboxyl and bromo-acetamide groups or combination of these molecules with for instance pyridine or alkyl groups. As carrier molecules, proteins, especially tetanus toxoid, KLH, diphtheria toxoid or poly amino acids, like polylysine can be used. Immunization with the oligosaccharides coupled to the said carrier molecules induces a T-cell mediated immune response.

Copolymerization can be done by the preparation of e.g. allylglycosides, preferably $-O-CH_2-CH=CH_2$, and successive coupling with vinylacetate or with other common polymerizable compounds known in the art. The effect is that two or preferably more oligosaccharide molecules are coupled to form larger agglomerates. In this case on immunization a T-cell independent B-cell response is obtained. Apart from the opportunity to mix different oligosaccharides, larger agglomerates also increase immunogenicity.

As outlined above, conjugation of the carbohydrate antigen to a protein carrier provides a T-cell dependent immunogen, whereas (co)polymerization of the carbohydrate antigen leads to a B-cell dependent immunogen. Hence, the desired type of immune response

can be selected by the way the carbohydrate antigen is engineered.

Also part of the invention are antibodies, fragments of antibodies, or analogues of antibodies or fragments, raised against the oligosaccharides according to the invention.

When polyclonal antibodies are desired, techniques for producing and processing polyclonal sera are known in the art (e.g. Mayer and Walter, eds, Immunochemical Methods in Cell and Molecular Biology, Academic Press, London, 1987). In short, a selected mammal, e.g. a rabbit is given (multiple) injections with an oligosaccharide of the invention, e.g. corresponding to about 20-80 μg of oligosaccharide per immunization. Immunization is carried out with an acceptable adjuvant, generally in equal volumes of immunogen and adjuvant. Acceptable adjuvants include Freund's complete, Freund's incomplete, alum-precipitate or water-in-oil emulsions, with a preference for Freund's complete adjuvant for the initial immunization. For booster immunization Freund's incomplete adjuvant is preferred. The initial immunization consists of the administration of approximately 1 ml emulsion at multiple subcutaneous sites on the backs of the rabbits. Booster immunizations utilizing an equal volume of immunogen are given at regular intervals, for example weekly or monthly intervals, and are continued until adequate levels of antibodies are present in an individual rabbits serum. Blood is collected and serum isolated by methods known in the art.

Monospecific antibodies to each of the immunogens are affinity purified from polyspecific antisera by a modification of the method of Hall et al. (Nature 311, 379-387 1984), prepared by immunizing rabbits as described above with the purified polymerized or carrier-linked oligosaccharides according to the

invention. Monospecific antibody as used herein is defined as a single antibody species or multiple antibody species with homogeneous binding characteristics for the relevant antigen. Homogeneous binding as used herein refers to the ability of the antibody species to bind to a specific antigen or epitope.

Monoclonal antibody reactive against one of the above-mentioned immunogens can be prepared by immunizing inbred mice, preferably Balb/c with the appropriate oligosaccharides (copolymerized or linked to a carrier)according to the invention by techniques known in the art (Kohler and Milstein, Nature 256; 495-497, 1975). Hybridoma cells are subsequently selected by growth in hypoxanthine, thymidine and aminopterin in an appropriate cell culture medium such as Dulbecco's modified Eagle's medium (DMEM). Antibody producing hybridomas are cloned, preferably using the soft agar technique of MacPherson, (Soft Agar Techniques, Tissue Culture Methods and Applications, Kruse and Paterson, eds., Academic Press, 276, 1973). Discrete colonies are transferred into individual wells of culture plates for cultivation in an appropriate culture medium. Antibody producing cells are identified by screening with the appropriate immunogen. Immunogen positive hybridoma cells are maintained by techniques known in the art. Specific monoclonal antibodies are produced by cultivating the hybridomas in vitro or preparing ascites fluid in mice following hybridoma injection by procedures known in the art.

In particular the invention is directed at an antibody directed against an oligosaccharide as described above. Because these antibodies interact with the oligosaccharides obtainable from the zona pellucida they block the sperm receptor ligand and thus they can be used for passive vaccination.

In particular such an antibody should be capable of inhibiting interaction between a sperm cell receptor and the corresponding oligosaccharide ligand on the oocyte.

When administering antibodies of animal origin to human beings it is possible that an anti-antibody response will occur. This can be prevented by administering humanized antibodies. One of the most common known methods in the art for humanizing antibodies is CDR-grafting (Jones et al., Nature 321, 522-525, 1986). Another way of avoiding the problem is by presenting only small parts of the antibody. Methods for producing such fragments that are still specific and have antibody activity are already described (Ukada et al., Mol. Immunol. 27, 25-35, 1990).

It is possible to produce vaccines with the oligosaccharides, conjugates or copolymers of the invention for active immunization. It is assumed that vaccines containing oligosaccharides of the invention have less side effects than vaccines containing the ZP3 polypeptide backbone. The ZP polysaccharides of the invention are subject to major structural changes during oocyte development (Shalgi, R. et al., Mol. Reproduc. Dev. 29, 365-372, 1991). They occur in the definite form when the oocyte is nearly matured and therefore they can be regarded as late antigens. It is conceivable that chances of adverse side-effects are smaller when antibodies are raised against ZP antigens that come to expression only in late stages of oocyte development. In this way the maturation of oocytes will be undisturbed. This also indicates that at the end of the treatment restoration to normal conditions will be faster than with antibodies against early antigens.
Due to its multispecies effects vaccination will be effective for all kinds of mammals, including pet animals like dogs, cats, guinea pigs, hamsters and the

like, and live stock, such as horses, sheep and cows and the like.

Depending on the protective capacity of the actively induced or passively given antibodies vaccinations have to be repeated to maintain the level of protection. A vaccination scheme can also be applied to postpone the fertile period to a later date.
Vaccination has to be repeated over a period of time depending on the characteristics of the ingredients of the vaccine. This is an improvement over 'every-day' or 'every-time' contraceptive methods used nowadays because it minimizes the chances of mistakes, misuse and inactivity.

The antibodies, fragments of antibodies and analogues of the antibodies or fragments can also be used as an immunochemical reagent. In this way they enable the detection of oligosaccharides on the oocyte, in vitro or in vivo. This can be useful for diagnosis of the presence of (nearly) mature oocytes, as is the case in, for instance, in vitro fertilization (IVF). Also the carbohydrates, equipped with a suitable label, can be used for detection and/or selection of sperm cells carrying receptors for these particular carbohydrate ligands.

The term "immunochemical reagent" signifies that the compounds mentioned above are bonded to a suitable support or are provided with a labelling substance.

The supports which can be used are, for example, the inner wall of a microtest well, a tube or capillary, a membrane, filter, test strip or the surface of a particle such as, for example, a latex particle, an erythrocyte, a dye sol, a metal sol or metal compound as sol particle.

Labelling substances which can be used are, inter alia, a radioactive isotope, a fluorescent compound, an enzyme, a dye sol, metal sol or metal compound as sol particle.

In a method for the detection of ligands or receptors, an immunochemical reagent according to the invention is used, which reagent is brought into contact with the test fluid, and the presence of immune complexes, formed between the immunochemical reagent and its counterpart in the test fluid, is detected and from this the presence of the ligand or the receptor can be derived. The immunochemical reaction which must take place when using these detection methods is preferably a sandwich reaction, an agglutination reaction, a competition reaction or an inhibition reaction.

A test kit according to the invention must contain, as an essential constituent, an immunochemical reagent such as described above. For carrying out a sandwich reaction, the test can consist of the - unlabelled -immunochemical reagent bonded to a solid support, for example the inner wall of a microtest well, it being possible to use a labelled immunochemical reagent for the detection.

For carrying out a competition reaction, the test kit can consist of the immunochemical reagent bonded to a solid support, a labelled antibody directed against this reagent then being used to compete with compounds in the test fluid.

In an agglutination reaction an immunochemical reagent bonded to particles or sols must be brought into direct contact with the test fluid in which the counterpart to the immunochemical reagents which is to be detected is present.

In the following examples the invention will be illustrated. The examples should therefore not be used as a limitation of the scope of the invention.

EXAMPLE 1

1. Isolation of zona pellucida glycoproteins.

Zonae pellucidae were obtained by homogenizing frozen-thawed porcine ovaries in a commercial meat grinder with copious amounts of ice-cold saline. This homogenate was sieved through two nylon screens of pore-size 500μm and 210μm to remove debris. The zonae were finally isolated from the filtrate on a 75 μm nylon screen. This crude zona preparation was purified by centrifugation in a discontinuous Percoll gradient (40, 20, 10% bottom-top) for 30 min. (2000 g, 25°C). The oocytes were collected from the 10-20% interface and were washed free from Percoll using phosphate buffered saline (PBS) and gently homogenized using a small glass homogenizer. Zonae were resuspended in PBS, heat solubilized at 78°C for 20 min. and finally ultracentrifuged (100,000 g, 90 min., 4°C). The clear supernatant was dialyzed against several changes of 0.1 M NH_4HCO_3 and lyophilized.

Sodiumdodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the heat solubilized zonae preparation gave rise to two diffuse bands in the molecular mass ranges of 90-85 kDa and 66-50 kDa, respectively. The lower band is derived from ZP2, ZP3α and ZP3ß yielding a diffuse pattern due to the extreme heterogeneity of the carbohydrate moieties of these glycoproteins.

2. Monosaccharide analysis.

TABLE 1. Molar carbohydrate composition of a heat solubilized zona pellucida preparation. Aver. is average of three independent assays.
s.e.m. is standard error of the mean.

Monosaccharide	aver.	s.e.m.
Fuc	1.1	0.22
Man[a]	3.0	0
Gal	8.3	0.61
GlcNAc[b]	10.8	0.36
GalNAc	1.1	0
NeuAc	1.1	0.29

[a] Mannose taken as 3.0

[b] Corrected for the amount of Asn-linked N-acetylglucosamine that is not cleaved under the conditions of methanolysis.

Monosaccharide analysis was carried out by gas chromatography on a capillary CP-Sil 5 WCOT fused silica column (25 m x 0.34 mm i.d., Chrompack) using a Hewlett Packard 5890 GC station. The trimethylsilylated methyl glycosides were prepared by methanolysis, N-(re)acylation and trimethylsilylation as reported (Kamerling and Vliegenthart, Cell. Biol. Monogr. 10, 95-125, 1982). The monosaccharide analysis data are presented in Table 1. The high molecular abundance of GlcNAc and Gal is indicative for the presence of carbohydrate chains containing multiple lactosamine repeats, which is in accordance with the data established by Nakano (Nakano et al., J. Biochem. 107, 144-150, 1990), Mori (Mori et al., Biochem. 30, 2078-2087, 1991), Nagdas (Nagdas et al., J. Cell Biol. 115, 47A, 1991), Yurewitz (Yurewitz et al. J. Biol. Chem. 262, 564-571, 1987; Yurewitz et al. Mol. Reprod. Dev. 30, 126-134, 1991) and Noguchi (Noguchi et al., Eur. J. Biochem. 204, 1089-1100, 1992). The presence of Man and GalNAc, respectively,

indicates that both N- and O-linked oligosaccharides occur.

3. Isolation of carbohydrate chains.

The heat solubilized zona pellucida preparation was subjected to the degradation and working-up procedure as schematically depicted in Fig. 1.
The N-linked carbohydrate chains were released from the protein moiety essentially as described earlier (J.B.L. Damm et al., Eur. J. Biochem. 180, 101-110, 1989). Briefly, 60 mg lyophilized heat solubilized ZP was dissolved to a concentration of 10 mg/ml in 50 mM Tris/HCl, pH 7.5, containing 50 mM EDTA, 1% (v/v) 2-mercaptoethanol and 1 mg SDS per mg ZP was added and the mixture was heated at 80°C for 3 min.
Subsequently, the mixture was cooled down to ambient temperature and 1 µl NP-40 per mg ZP was introduced. After thoroughly mixing, 1 U PNGase-F (peptide-N^4-(N-acetyl-ß-glucosaminyl)asparagine amidase-F from *Flavobacterium meningosepticum* (E.C. 3.5.1.52, obtained from Boehringer Mannheim) per mg ZP was added and the mixture was incubated at ambient temperature. After 4 hours, a fresh aliquot of 1 U PNGase-F per mg ZP was introduced and the incubation was continued for 16 hours. Completeness of liberation of the N-linked chains was checked by SDS-PAGE. The sample was fractionated by gelpermeation chromatography on a Bio-Gel P-100 column (1.5 x 57 cm, 200-400 mesh, BioRad) using 25 mM NH_4HCO_3 as eluent. The carbohydrate-positive fractions (detection by orcinol/H_2SO_4 assay) were pooled and lyophilized. Subsequently, remaining detergents were removed by affinity chromatography over an ExtractiGel-D column (8 x 1 cm, Pierce), using 25 mM NH_4HCO_3 as eluent and the material in the run-through fraction was desalted by gelpermeation chromatography

on a BioGel P-2 column (18 x 1 cm, 200-400 mesh, BioRad) using Milli-Q water as eluent.
Part of the O-glycoprotein-SDS complex (denoted as O-linked (red.) in Fig. 1), eluting in the void volume of the P-100 column, was treated with alkaline borohydride as described (J.B.L. Damm et al., Glycoconj. J. 4, 129-144, 1987) to release the O-linked chains in the form of alditols. The ß-elimination products were subjected to ExtractiGel-D, Dowex-H^+ and BioGel P-2 chromatography, respectively, as described for the released N-glycosidic chains, and lyophilized.
A second part of the O-glycoprotein-SDS complex (denoted as O-linked in Fig. 1) was subjected to pronase treatment. Further purification of the glycopeptide was performed by liquid chromotography using a BioGel P-2 column.

4. Structural analysis of the O-linked oligosaccharides

The pool of O-linked oligosaccharide alditols (denoted as O-linked red. in Fig. 1) was subjected to a working-up procedure as outlined in Fig. 3. Anion-exchange chromatography over Mono Q (Fig. 4) gave rise to a fraction denoted O-neutral, containing uncharged carbohydrates, and a very broad region, denoted O-charged, containing carbohydrates of increasing charge. The fraction containing the uncharged O-glycosidic carbohydrate chains was applied to a small column of Dowex 50W-X8, H^+ form (6 x 0.5 cm, 100-200 mesh, Fluka). The column was eluted with 6 ml 0.01M formic acid, and the eluate was lyofilized, redissolved and subfractionated on a Lichrosorb-NH_2 10μ column (25 x 0.46 cm, Chrompack) (shown in Fig. 5) essentially as described (J.B.L. Damm et al., Eur. J. Biochem. 189, 175-183, 1989). The carbohydrate containing Lichrosorb-NH_2 fractions were desalted on BioGel P-2 and lyophilized. The O-

charged fractions were pooled and subjected to gelpermeation chromatography.

The pool of negatively charged O-glycosidic carbohydrate chains was fractionated on a BioGel P-4 column (150 x 1.15 cm, 200-400 mesh, BioRad) using 100 mM NH_4HCO_3 as eluent. The column was calibrated with an endo-ß-galactosidase digest of bovine keratansulfate (Sigma), containing ZP-like sulfated lactosaminoglycan fragments. NH_4HCO_3 was removed from the fractions by lyophilization. The eluate was divided in 8 fractions denoted P4.1 - P4.8 (Fig. 6). Fractions P4.1 - P4.3 were carbohydrate positive (orcinol/H_2SO_4 assay). Fraction P4.1 was rechromatographed on a BioGel P-6 column (135 x 2.2 cm, 200-400 mesh, BioRad) using the same eluent. This yielded a poor separation (Fig. 7) except for the presence of a distinct void volume peak. The fractions were denoted P6.1 - P6.7 as denoted in Fig. 7.

^{1}H-NMR spectroscopy revealed in each of the fractions P6.1 - P6.7, P4.2 and P4.3 the presence of a complex mixture of sulfated and/or sialylated lactosaminoglycan type oligosaccharides. To remove most of the contaminants these fractions were passed over a small Dowex H^+ 50W-X8 cation exchange resin (BioRad), using 10 mM formic acid as eluent. The run-through fraction was collected and lyophilized. Subsequently the fractions P6.2-P6.7, P4.2 and P4.3 were subfractioned on a Mono Q HR 5/5 anion-exchange column (Pharmacia) according to charge as described (J.B.L. Damm et al., Glycoconj. J. 4, 129-144, 1987), except that the column was eluted with a discontinuous NaCl gradient from 0 to 1 M as indicated in Fig. 8A-8H.

The carbohydrate-containing Mono Q fractions, monitored at 214 nm, were collected, desalted on BioGel P-2 and lyophilized. The fractionation scheme

is indicated in Fig. 3. Some of the Mono Q fractions were further fractionized by high-pH anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) on a CarboPac PA-1 pellicular anion-exchange column (25 x 0.9 cm, Dionex) as described by K. Hard et al., Eur. J. Biochem. 209:895-915 (1992) (see Fig. 10).

5. 500 MHz ^{1}H-NMR spectroscopy

Prior to ^{1}H-NMR spectroscopic analysis, the desalted samples were exchanged twice with ^2H_2O. Finally, samples were dissolved in 99.96 atom % ^2H_2O (MSD isotopes). ^{1}H-NMR spectra were recorded at 500 MHz using a Bruker AMX-500 spectrometer (Bijvoet Center, Dept. NMR spectroscopy, Utrecht University, The Netherlands) at a probe temperature of 22°C. Chemical shifts are expressed in ppm by reference to internal acetone (δ=2.225ppm in ^2H_2O at 22°C) (Vliegenthart et al., Adv. Carbohydr. Chem. Biochem. 41, 209-374, 1983). Typically, one dimensional (1D) spectra were recorded with a spectral width of 5000 Hz, collecting 128-2000 free induction decays (FID's) of 8K or 16K complex data points. Suppression of the residual water signal was achieved by applying a pulse sequence based on the WEFT sequence (Hård et al, unpublished results). The resolution of 1D spectra was enhanced by Lorentzian-to-Gaussian transformation and the final spectra were baseline corrected with a polynomal function when necessary.
For the two-dimensional homonuclear Hartmann-Hahn (2D HOHAHA) spectra (Bax and Davis, J. Magn. res. 65, 355-360, 1985) a 100-120 ms MLEV-17 mixing sequence was used. The 90° ^{1}H pulse width was adjusted to 26 μs and the spectral width was 3000-3500 Hz in both dimensions. The HO^{2}H signal was suppressed by presaturation for 1.0 s during the relaxation delay.

In total 296-512 spectra of 2048 datapoints with 56-128 scans per t_1 value were recorded. 2D-NMR data were processed on a VAX^R station 3100 using TRITON software (Bijvoet Center, Dept. of NMR Spectroscopy, Utrecht University, The Netherlands). The time domain data were multiplied with a phase shifted sine bell. After Fourier transformation, the resulting data set of 1024 x 2048 data points was baseline corrected in both frequency domains with a fourth order polynomal fit when necessary.

^{1}H-NMR spectroscopy revealed in each of the isolated carbohydrate fractions the presence of (sulfated and/or sialylated) lactosaminoglycan type oligosaccharides. The established structures are compiled in Table 2. In summary, the 1D and 2D ^{1}H-NMR spectra of the major Mono Q fractions show that the oligosaccharides share common characteristics. The core structure is in most cases Galß1-4GlcNAcß1-3Galß1-3GalNAc-ol. The core can be elongated by extra N-acetyllactosamine units (up to >10) and in that case almost all extra GlcNAc residues are 6-O-sulfated. Furthermore, a small portion of the oligosaccharides contains a 6-O-sulfated core GlcNAc residue (GlcNAc^C). When no additional N-acetyllactosamine units are present, the GalNAc-ol can be substituted by α_{2-3} or α_{2-6} linked NeuGc or NeuAc. The elongated chains can be terminated by a Gal residue (minor) or by α_{2-3} linked NeuAc/Gc (major). No indications have been found for the presence of branched poly-N-acetyllactosamine chains or sulfated Gal residues.

This general concept for the structure of the pZP O-linked oligosaccharides is depicted in Fig. 9.

In Table 2 the individual, purified O-linked structures from porcine ZP are listed. Table 3 gives the ^{1}H chemical shifts of the structural-reporter-group protons

of the constituent monosaccharides of some representative pZP-derived O-linked oligosaccharides. The oligosaccharides P6.7Q4 and P6.6Q7 contain either a NeuGc (denoted P6.7Q4A and P6.6Q7A) or a NeuAc residue (denoted P6.7Q4B and P6.6Q7B).

Table 2: O-linked strctures from porcine ZP

N.2A: Galß1-4GlcNAcß1-3GalNAc-ol

N.2B: GlcNAcß1-3Galß1-3GalNAc-ol

N.4: Galß1-4GlcNAcß1-3Galß1-3GalNAc-ol

N.6: Galα1-3Galß1-4GlcNAcß1-3Galß1-3GalNAc-ol

N.7: Galß1-4GlcNAcß1-3Galß1-4GlcNAcß1-3Galß1-3GalNAc-ol

N.9: $[Galß1\text{-}4GlcNAcß1\text{-}3]_2Galß1\text{-}4GlcNAcß1\text{-}3Galß1\text{-}3GalNAc\text{-}ol$

P4.3Q2: NeuAc/Gcα2-3Galß1-3GalNAc-ol

P4.3Q3.5: Galß1-4GlcNAcß1-3Galß1-3GalNAc-ol
|
$6SO_4$

P4.2Q3.2: NeuAcα2-6Galß1-4GlcNAcß1-3Galß1-3GalNAc-ol

P4.2Q3.6: NeuGcα2-6Galß1-4GlcNAcß1-3Galß1-3GalNAc-ol

Neu5Gcα2-6
\
P4.2Q3.7: Galß1-4GlcNAcß1-3Galß1-3GalNAc-ol

P4.2Q4: Galß1-4GlcNAcß1-3Galß1-4GlcNAcß1-3Galß1-3GalNAc-ol
|
$6SO_4$

P4.2Q7+ NeuAC/Gcα2-6
\
P6.7Q3: NeuAc/Gcα2-3Galß1-3GalNAc-ol

P6.7Q4: NeuAc/Gcα2-3Galß1-4GlcNAcß1-3Galß1-4GlcNAcß1-3Galß1-3GalNAc-ol
|
$6SO_4$

P6.7Q5: $[Galß1\text{-}4GlcNAcß1\text{-}3]_2Galß1\text{-}4GlcNAcß1\text{-}3Galß1\text{-}3GalNAc\text{-}ol$
|
$6SO_4$

P6.6Q7: NeuAc/Gcα2-3[Galß1-4GlcNAcß1-3]$_2$Galß1-4GlcNAcß1-3Galß1-3GalNAc-ol
$6SO_4$

P6.6Q8: [Galß1-4GlcNAcß1-3]$_3$Galß1-4GlcNAcß1-3Galß1-3GalNAc-ol
$6SO_4$

P6.6Q10: NeuAc/Gcα2-3[Galß1-4GlcNAcß1-3]$_3$Galß1-3GalNAc-ol
$6SO_4$

P6.5Q5: NeuAc/Gcα2-3[Galß1-4GlcNAcß1-3]$_3$Galß1-4GlcNAcß1-3Galß1-3GalNAc-ol
$[6SO_4]_2$

P6.5Q8: NeuAc/Gcα2-3[Galß1-4GlcNAcß1-3]$_3$Galß1-4GlcNAcß1-3Galß1-3GalNAc-ol
$6SO_4$

P6.5Q9: [Galß1-4GlcNAc1-3]$_4$Galß1-4GlcNAcß1-3Galß1-3GalNAc-ol
$6SO_4$

P6.4Q9: Neu5Ac/Gcα2-3[Galß1-4GlcNAcß1-3]$_4$Galß1-4GlcNAcß1-3Galß1-3GalNAc-ol
$6SO_4$

P6.4Q7: Neu5Ac/Gcα2-3[Galß1-4GlcNAcß1-3]$_4$Galß1-4GlcNAcß1-3Galß1-3GalNAc-ol
$[6SO_4]_3$

Table 3. ^{1}H-Chemical shifts of structural-reporter-group protons of the constituent monosaccharides of O-linked oligosaccharides derived from porcine Zona Pellucida glycoprotein. Chemical shifts are given at 22 °C and were measured in ^2H_2O relative to acetone (δ 2.225). For structures and numbering of the residues, see text.

Reporter group	Residue	Chemical shift (ppm) in					
		P4.2.4	P6.7.4A	P6.7.4B	P6.7.5	P.6.6.7A	P.6.6.7B
GalNAc-ol	H-2	4.401	4.399	4.399	4.400	4.396	4.396
	H-3	4.049	4.048	4.048	4.048	4.049	4.049
	H-4	3.490	3.489	3.489	3.489	3.495	3.495
	H-5	4.186	4.188	4.188	4.188	4.185	4.185
	NAc	2.047	2.047	2.047	2.046	2.047	2.047
Gal	H-1	4.460	4.459	4.459	4.459	4.461	4.461
	H-4	4.126	4.126	4.126	4.125	4.125	4.125
GlcNAc[c]	H-1	4.679	4.677	4.677	4.677	4.683	4.683
	H-6	3.950	3.949	3.949	3.949	3.954	3.954
	NAc	2.038	2.037	2.037	2.037	2.037	2.037
Gal[c]	H-1	4.470	4.469	4.469	4.468	4.470	4.470
	H-4	4.189	4.194	4.194	4.186	4.191	4.191
GlcNAc[i]	H-1	-	-	-	4.711	4.712	4.712
	H-6	-	-	-	4.397	4.404	4.404
	H-6'	-	-	-	4.297	4.285	4.285
	NAc	-	-	-	2.037	2.034	2.034
Gal[i]	H-1	-	-	-	4.507	4.506	4.506
	H-4	-	-	-	4.186	4.191	4.191
GlcNAc[e]	H-1	4.716	4.707	4.707	4.711	4.706	4.706
	H-6	4.395	4.403	4.403	4.376	4.387	4.387
	H-6'	4.314	4.314	4.314	4.328	4.325	4.325
	NAc	2.032	2.029	2.029	2.028	2.028	2.028
Gal[e]	H-1	4.523	4.601	4.601	4.528	4.604	4.604
	H-3	n.d.[a]	4.132	4.122	n.d.	4.132	4.122
	H-4	3.926	3.963	3.968	3.923	3.965	3.971
NeuGc	H-3a	-	1.819	-	-	1.819	-
	H-3e	-	2.767	-	-	2.768	-
	NGc	-	4.116	-	-	4.117	-
NeuAc	H-3a	-	-	1.804	-	-	1.803
	H-3e	-	-	2.746	-	-	2.748
	NAc	-	-	2.029	-	-	2.028

[a] n.d., not determined

EXAMPLE 2

1. Preparation of pZP-derived O-glycosidic aminocarbohydrate chains

Porcine ZP (7.3 mg) was N-deglycosylated as described in Example 1. The effectiveness of N-deglycosylation was checked by SDS-PAGE of the sample before and after PNGase-F treatment. The N-deglycosylated material (viz. the O-glycoprotein) was lyophilized and redissolved in 1.5 mL 0.1 mol/L NH4Ac, pH 8.0, containing 15 mmol/L CaCl2 and 0.02% (m/v) NaN3 (enzyme buffer). Pronase (30 U, Streptococcus griseus, Boehringer) was dissolved in 160 µL enzyme buffer and preincubated for 1 h at 40 °C. Aliquots of 25, 25, 10 and 10 µL pronase solution were added to the O-glycoprotein at 0, 5, 24 and 48 h, respectively. Between additions the pronase solution was kept at -20 °C and during proteolysis the sample solution was kept at pH 8 by addition of 0.2 mol/L NaOH. After 52 h the sample solution was heated at 100 °C for 5 min to inactivate pronase. The effectiveness of the pronase digestion was examined by SDS-PAGE followed by silver staining of the gel. The digest was desalted by gelfiltration over BioGel P-2 as decribed in Example 1 and lyophilized. Subsequently, the sample was redissolved in 500 µL Milli Q and passed over an Extracti-Gel D column, using 25 mmol/L NH4HCO3 as eluent. The carbohydrate-positive fraction (orcinol/H2SO4 spot-test) was collected and lyophilized.

2. Conjugation of pZP-derived O-glycosidic aminocarbohydrate chains to KLH

The pZP-derived O-glycosidic aminocarbohydrate chains (± 500 µg) were dissolved in 500 µL PBS. 100 µL of

this solution was kept apart for determination of the bioactivity in the hZBA and a profile analysis of the carbohydrate chains by capillary electrophoresis. To the remaining solution (400 µL, 400 µg aminocarbohydrate chains) 800 µL PBS, containing 1 mg/mL KLH was added. Next, 100 µL 60 mmol/L glutardialdehyde was introduced and the mixture was incubated at RT for 1 h. Subsequently, 40 µL 0.5 mol/L glycine, pH 8 was added and the incubation was continued for 30 min at RT. The KLH-carbohydrate conjugate was desalted by gelfiltration over a disposable PD-10 column using PBS as eluent, followed by dialysis against Milli Q. The desalted product was lyophilized and stored at -20 °C till use.

3. Preparation of antibodies against pZP-derived O-glycosidic aminocarbohydrate chains

Polyclonal antisera were raised against KLH and the conjugates of pZP-derived O-glycosidic carbohydrate chains with KLH. KLH and the carbohydrate-KLH conjugate were both dissolved in Milli Q to a concentration of 1 mg/ml. Subsequently, an amount of carbohydrate-KLH conjugate corresponding to ±15 µg carbohydrate material or an amount of KLH corresponding to the amount of KLH in the conjugate were mixed with 50 µL complete (first immunization) or incomplete (boosters) Freunds adjuvant. In each case the total volume was brought to 100 µL by addition of PBS. For immunization male Balb/c mice were used and the preparations were injected subcutaneously at 0, 4 and 8 weeks.

4. Activity of the antibodies against pZP-derived O-linked oligosaccharides

Mice were bled at 8 weeks, the titer of these antibodies against pZP was determined in an enzyme immnunoassay. Plates were coated with 25 ng pZP per well overnight in a 0.1 M carbonate buffer. After several washes with PBS-Tween, 50 µl of the diluted mice sera were added for 1 hour, after several washes 100 µl of an rabbit antibody labelled with HRP diluted 1000x was added for 1 hour, whereafter again several washes were performed. The binding of the HRP labeled antibody is detected by the development of a substrate (for instance TMBS) whereafter the colour development is measured at 450 nm.

As can be seen from Fig. 11, the antibodies against the O-linked carbohydrates of pZP give much higher signal than the antibodies against KLH.

Subsequently we have tested the effect of coating the ZP with antibodies against the O-linked carbohydrates of pZP on the binding of sperm to the ZP. Therefore porcine oocytes were coated for 1 hour with the antibodies againstthe O-linked carbohydrates of pZP coupled to KLH or as a control with antibodies against KLH. Subsequently porcine sperm were introduced (50 µl of 1 million/ml, which have been capacitaed for 1 hour) and the oocytes and sperm were incubated for 1 hour. Bound sperm were treated as discribed in Example 3.

As can be seen in Figure 12 the number of sperm bound to the oocytes is reduced in case the oocytes had been coated with the antibodies against the O-linked carbohydrates of pZP.

EXAMPLE 3

1. Porcine and human zona binding assays (pZBA and hZBA)

Porcine or human (salt stored, unfertilized) eggs were rinsed several times in an appropriate sperm-buffer. Eggs and sperm were preincubated separately with buffer (negative control), zona pellucida protein (positive control) or carbohydrate chains for 60 min at 37°C (pZBA and hZBA), followed by coincubation of eggs and sperm for 60 min at 37°C (pZBA) or for 240 min (hZBA). Loosely adherent and firmly attached spermatozoa were separated on a dextran gradient. Then firmly bound spermatozoa were fixed and stained with buffer, containing 1% glutaraldehyde and 2 µg/ml Hoechst H33258 (a compound staining DNA), and the number of bound spermatozoa was counted with a fluorescence microscope.

In Fig. 2A the effects of the zona pellucida starting material, the O-glycoprotein and the ensambles of released N- and O-linked oligosaccharides were tested in the pZBA in four independent experiments. With the zona pellucida control a dose-dependent inhibition (45% at 5 µg/ml, 75% at 50 µg/ml, expressed as percentage of control) is obtained. A nearly identical effect is noticed for the O-glycoprotein (tested at 5 and 50 µg/ml) and the pool of released O-linked carbohydrated chains (tested at 1 and 10 µg/ml, equivalent to approx. 10 and 100 µg/ml zona pellucida protein, respectively). The pool of N-linked carbohydrate chains does not significantly inhibit sperm-oocyte binding at either test concentration. From Fig. 2B it is evident that the O-glycoprotein and the pool of released O-linked oligosaccharides also inhibit binding of human sperm

to human egg cells. The inhibition is not due to reduced sperm motility as can be seen in the lower diagram of Fig. 2B.

The charged O-linked carbohydrate chains were subjected to the working-up procedure discussed previously and the structures of the purified oligosaccharides were determined by 500 MHz 1H-NMR spectroscopy. Subsequently, the capacity of several of the purified and identified O-linked oligosaccharides to interfere with human sperm-oocyte binding was tested. The experimental data as shown in Fig. 13A, strongly suggest that oligosaccharides consisting of the core tetrasaccharide and at least one sulfated, non-sialylated N-acetyllactosamine unit inhibit (>70% at 5 μM) the binding of human sperm to human eggs. It appears that in the pZBA sialylation abolishes the bioactivity. In the hZBA the effect of sialylation is less clear.

Furthermore, it was established by endo-ß- galactosidase treatment of the parent oligosaccharides, that neither the core unit, nor a sulfated N-acetyllactosamine fragment as such can inhibit sperm-oocyte binding (Fig. 13B).

LIST OF ABBREVIATIONS AND LEGENDS TO THE FIGURES

Abbreviations:

Asn	Aspargine
CDR	Complementarity determining region
EDTA	Ethylenediaminetetraacetic acid
Fuc	Fucose
Gal	Galactose
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
Glc	Glucose
HPLC	High Performance Liquid Chromatography
HRP	Horse radish peroxidase
hZBA	human zona binding assay
KLH	Keyhole limpet hemocyanin
Man	Mannose
NAc	N-acetyl group
NeuAc	N-acetyl neuraminic acid
NeuGc	N-glycolyl neuraminic acid
NMR	Nuclear Magnetic Resonance
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pZBA	porcine zona binding assay
pZP	porcine zona pellucida protein
SDS	sodium dodecyl sulphate
s.e.m.	standard error of the mean
SPDP	N-succinimidyl-3-(2-pyridyldithio)-proprionate
ZP	Zona Pellucida protein

Fig. 1.

Purification scheme of porcine zona pellucida (pZP) carbohydrate chains.

Fig. 2A.

Effect of pZP, O-glycoprotein (O-GP), N-linked oligosaccharides (N) and O-linked oligosaccharide alditols (O) on porcine sperm-egg binding in four independent assays. Results are expressed as percentage of control (average ± standard error of the mean). The pZP and the O-glycoprotein were tested at 5 and 50 µg/ml. The N- and O-linked carbohydrate chains were tested at a concentration equivalent to 10 and 100 µg pZP/ml.

Fig. 2B.
Effect of pZP O-glycoprotein (ZP1 O-Glyco, approx. 50 μg) and pZP O-linked oligosaccharides (ZP1 ß-elim, approx. 10 μg) on human sperm-egg binding (top) and sperm motility (bottom).

Fig. 3.
Purification scheme of the pZP O-linked oligosaccharides obtained by alkaline borohydride treatment of the N-deglycosylated O-glycoprotein.

Fig. 4.
Fractionation of the pZP O-linked oligosaccharides obtained by alkaline borohydride treatment by High Pressure Liquid Chromatography (HPLC) on Mono Q. Y-axis: U.V. absorbance at 214 nm.

Fig. 5.
Subfractionation of the pZP O-linked oligosaccharides on a Lichrosorb-NH_2 10μ column (25 x 0.46 cm, Chrompack)

Fig. 6.
Gelpermeation chromatography of negatively charged O-glycosidic carbohydrate chains from pZP. Eight fractions denoted P4.1 to P4.8 are obtained.

Fig. 7.
Chromatogram of fraction P4.1 (Fig. 6) on BioGel P-6 column. Seven fractions denoted P6.1 to P6.7 are obtained.

Fig. 8.
A. Chromatogram (Mono Q) of fraction P4.3.
B. Chromatogram (Mono Q) of fraction P4.2.
C. Chromatogram (Mono Q) of fraction P6.
D. Chromatogram (Mono Q) of fraction P6.6.
E. Chromatogram (Mono Q) of fraction P6.5.

F. Chromatogram (Mono Q) of fraction P6.4.
G. Chromatogram (Mono Q) of fraction P6.3.
H. Chromatogram (Mono Q) of fraction P6.2.

Fig. 9.
General structure of the O-linked carbohydrate chains derived from pZP.

Fig. 10.
Chromatogram of the further fractionation of some Mono Q fractions by HPAEC.

Fig. 11.
Binding of mice antibodies (AB) against O-linked sugars and KLH to pZP coated plates.

Fig. 12.
Effect of antibodies against O-linked sugars of pZP on porcine sperm binding to ZP.

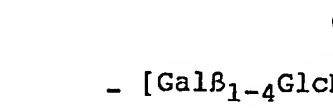
Fig. 13A.
Effect of four pZP-derived carbohydrate chains on human sperm-egg binding. The O-linked oligosaccharides were released from N-deglycosylated pZP and then fractionated as indicated in Fig. 3. Results are expressed as sperm cells bound per square mm hZP (average ± s.e.m.). C is Control milli Q water diluted in the same way as the dilutions of oligosaccharides. AB54 is positive control of polyclonal rabbit serum antibodies obtained from immunization with human eggs. Nr. 1 is the oligosaccharide denoted P4.2 Q4 in Table 2, tested in a concentration of 5.32 μM, nr. 2 is P6.7 Q4 in a concentration of 2.12 μM, nr. 3 is P6.7 Q5 in a concentration of 2.66 μM and nr. 4 is P6.6 Q7 in a concentration of 3.2 μM .

Fig. 13B.

Effect of five pZP-derived carbohydrate chains on human sperm-egg binding. All carbohydrate chains were tested at a concentration of 5 μm. Further details in Fig. 13A. Nr. 1 is the oligosaccharide denoted P4.3 Q6 in Table 2, nr. 2 is P6.7 Q3, nr. 3 is P6.6 Q8, nr. 4 is P6.5 Q8 and nr. 5 is a mixture of oligosaccharides prepared by endo-ß-galactosidase digestion of an ensamble of pZP O-linked oligosaccharides.

CLAIMS

1. Oligosaccharides, characterized in that they comprise the structure:



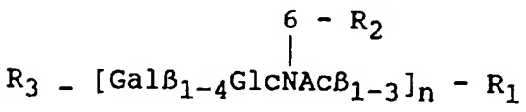
with n = 1-10

R_1= $Gal\beta_{1-3}GalNAc$, and

R_2= OH or OSO_3^- or $\alpha_{2-6}NeuAc$

or pharmaceutically acceptable salts thereof.

2. Oligosaccharides, characterized in that they have the following structure:



with n = 1-10

R_1= $Gal\beta_{1-3}GalNAc_{\alpha/\beta 1}-R_4$ or $Gal\beta_{1-3}GalNAc$-ol

R_2= OH or OSO_3 or $\alpha_{2-6}NeuAc$

R_3= H, $6SO_4$, $4SO_4$, $3SO_4$, $\alpha_{2-3}NeuAc$, $\alpha_{2-3}NeuGc$, $\alpha_{2-6}NeuAc$, $\alpha_{2-6}NeuGc$, GlcNAc, GlcNAc-$_6SO_4$,

R_4 = H or a group for polymerization or for conjugation to an immnuogenic carrier.

3. Oligosaccharides according to any of the claims 1 and 2 for contraceptive treatment.

4. Use of the oligosaccharides according any of the claims 1 and 2 as contraceptive.

5. Conjugates and clusters comprising the oligosaccharides according to any of the claims 1 and 2 and a carrier molecule.

6. Copolymers of the oligosaccharides according to any of the claims 1 and 2 with polymerisable compounds.

7. Monoclonal antibodies, fragments of antibodies, or analogues of said antibodies or fragments, raised against oligosaccharides according to any of the claims 1 and 2.

8. Vaccine for contraceptive use, characterized in that it comprises one or more compounds selected from the group consisting of oligosaccharides according to any of the claims 1 and 2, conjugates according to claim 5, copolymers according to claim 6 or monoclonal antibodies according to claim 7, and suitable incipients.

9. Pharmaceutical composition, characterized in that it comprises one or more compounds selected from the group of oligosaccharides according to any of the claims 1 and 2, conjugates according to claim 5, copolymers according to claim 6 or antibodies according to claim 7, and suitable incipients.

10. Immunodiagnostic reagent comprising an oligosaccharide according to any of the claims 1 and 2 or an antibody, fragment of antibody, or an analogue of said antibody or fragment according to claim 7.

Fig. 1

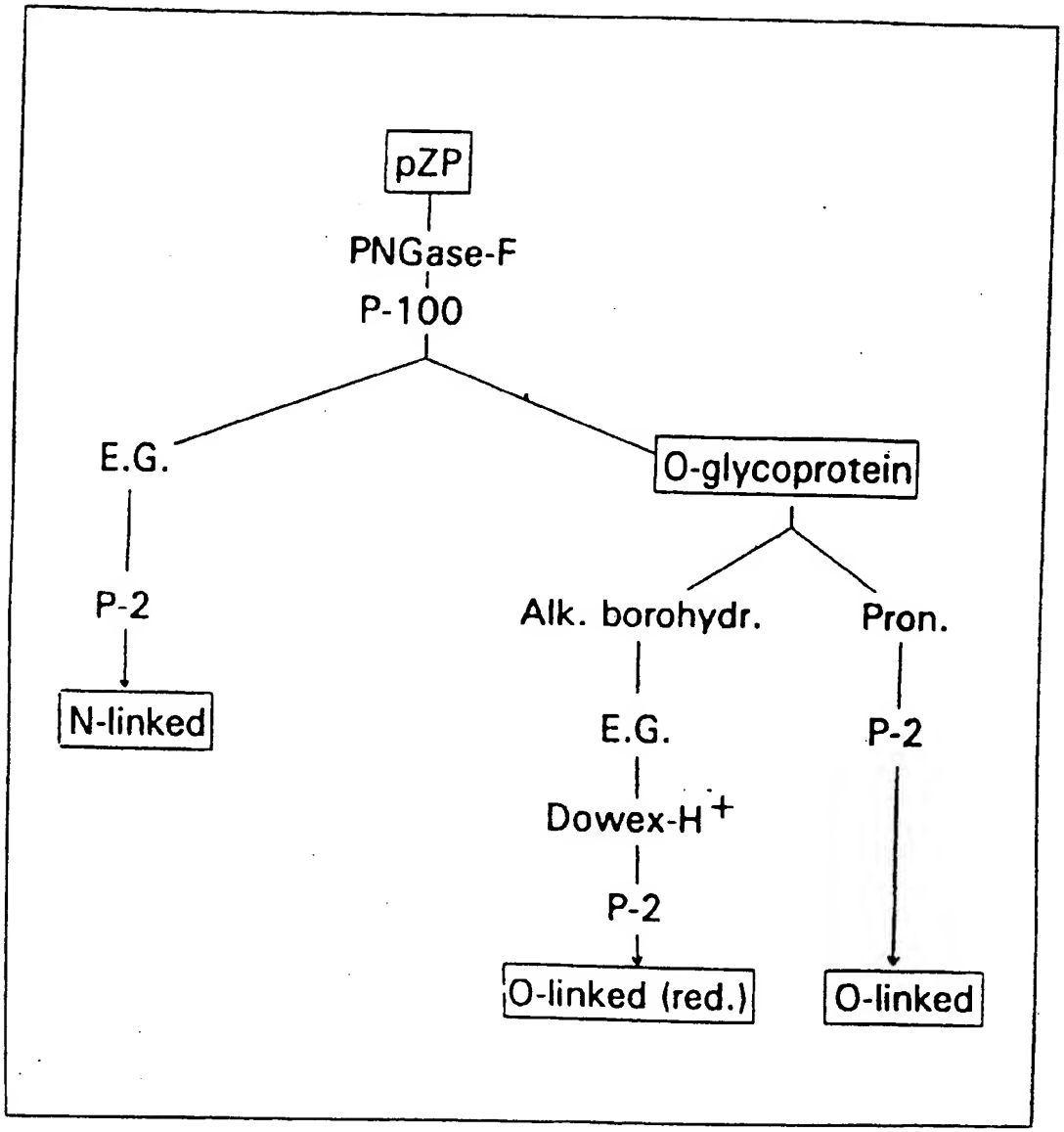

pZP
PNGase-F
P-100
E.G.
O-glycoprotein
P-2
Alk. borohydr.
Pron.
N-linked
E.G.
P-2
Dowex-H+
P-2
O-linked (red.)
O-linked

Fig. 2A

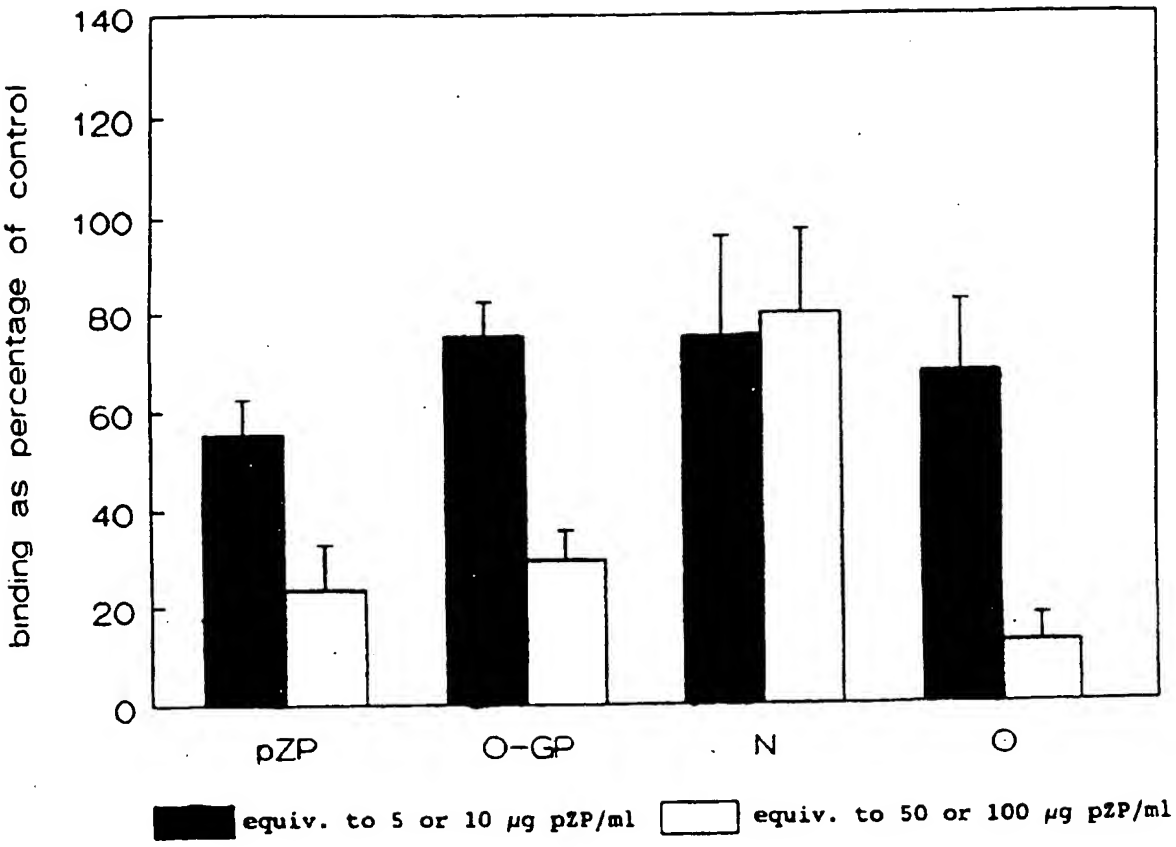
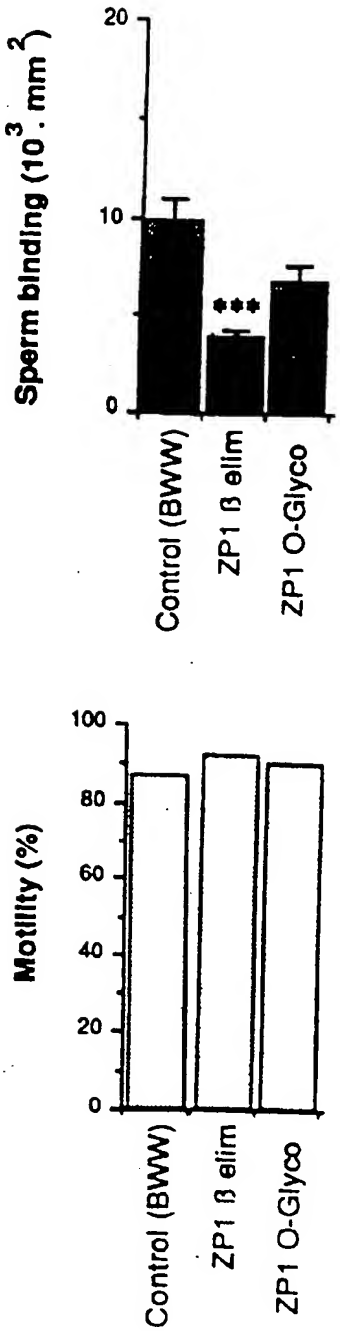

binding as percentage of control
140
120
100
80
60
40
20
0
pZP
O-GP
N
O
equiv. to 5 or 10 µg pZP/ml
equiv. to 50 or 100 µg pZP/ml

Fig. 2B


Sperm binding (10^3. mm^2)
20
10
0
***
Control (BWW)
ZP1 ß elim
ZP1 O-Glyco
Motility (%)
100
80
60
40
20
0
Control (BWW)
ZP1 ß elim
ZP1 O-Glyco

WO 94/19361 PCT/EP94/00569

Fig. 3

Separation scheme of the intact O-linked carbohydrate chains.

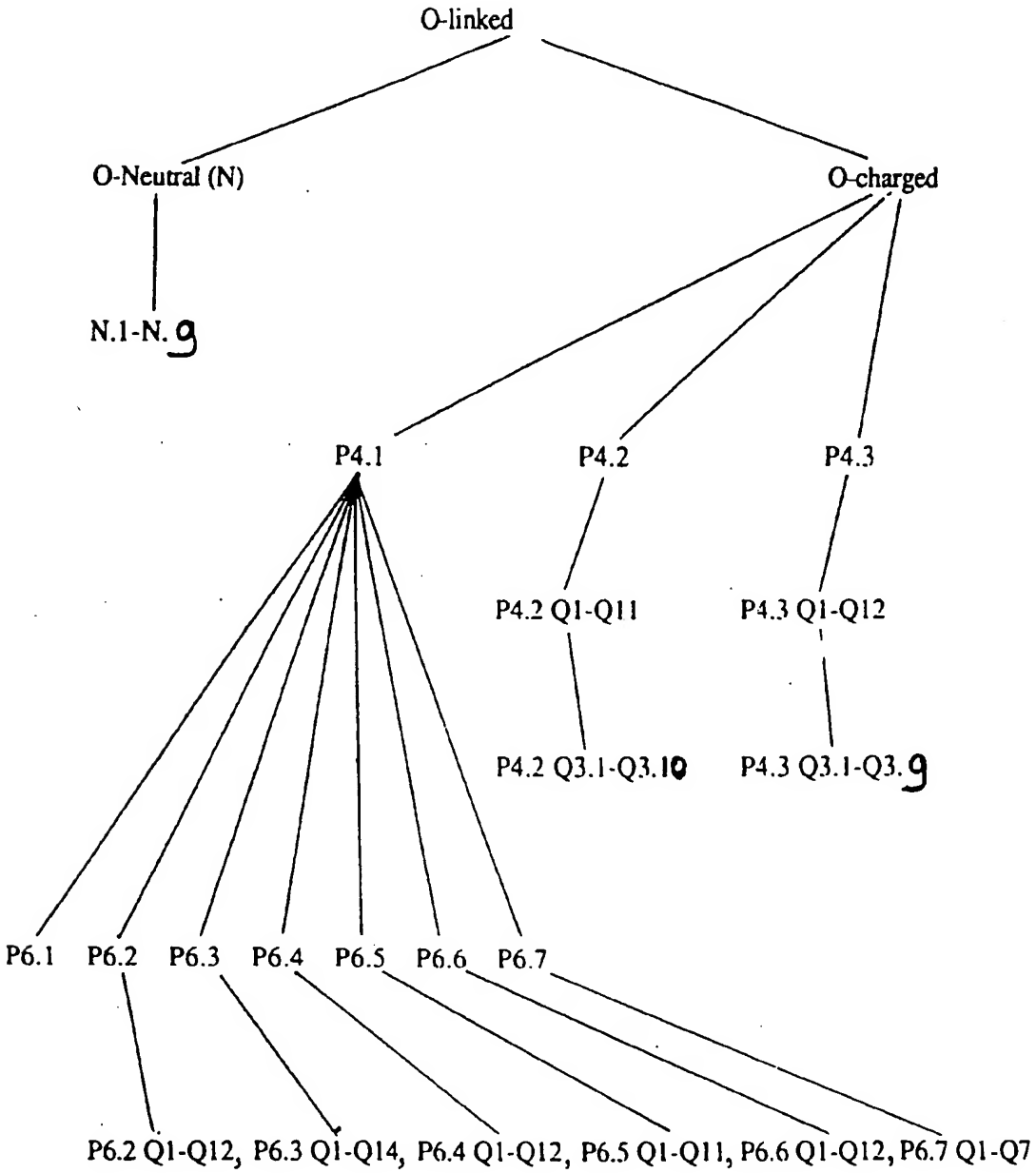
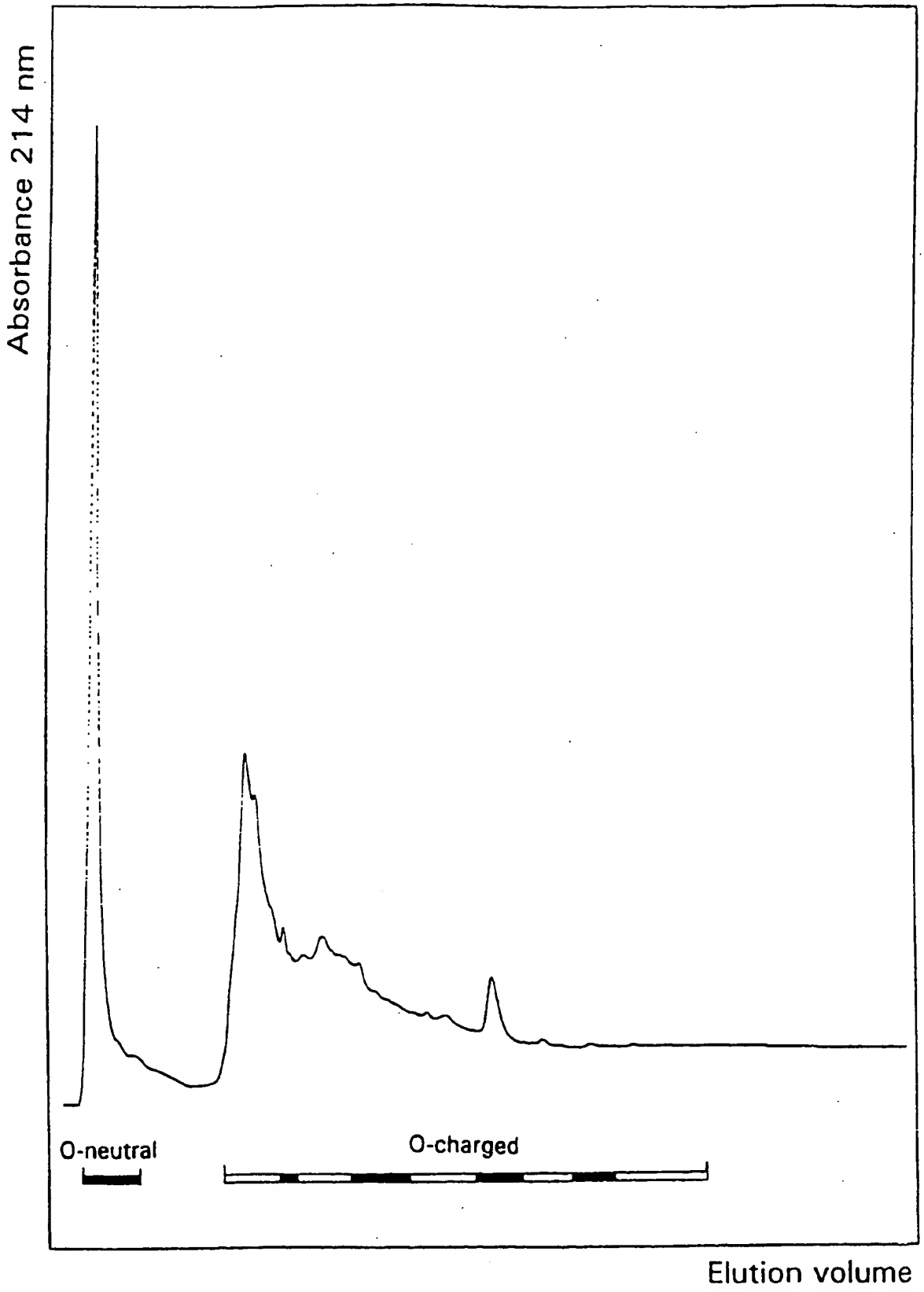


Fig. 4



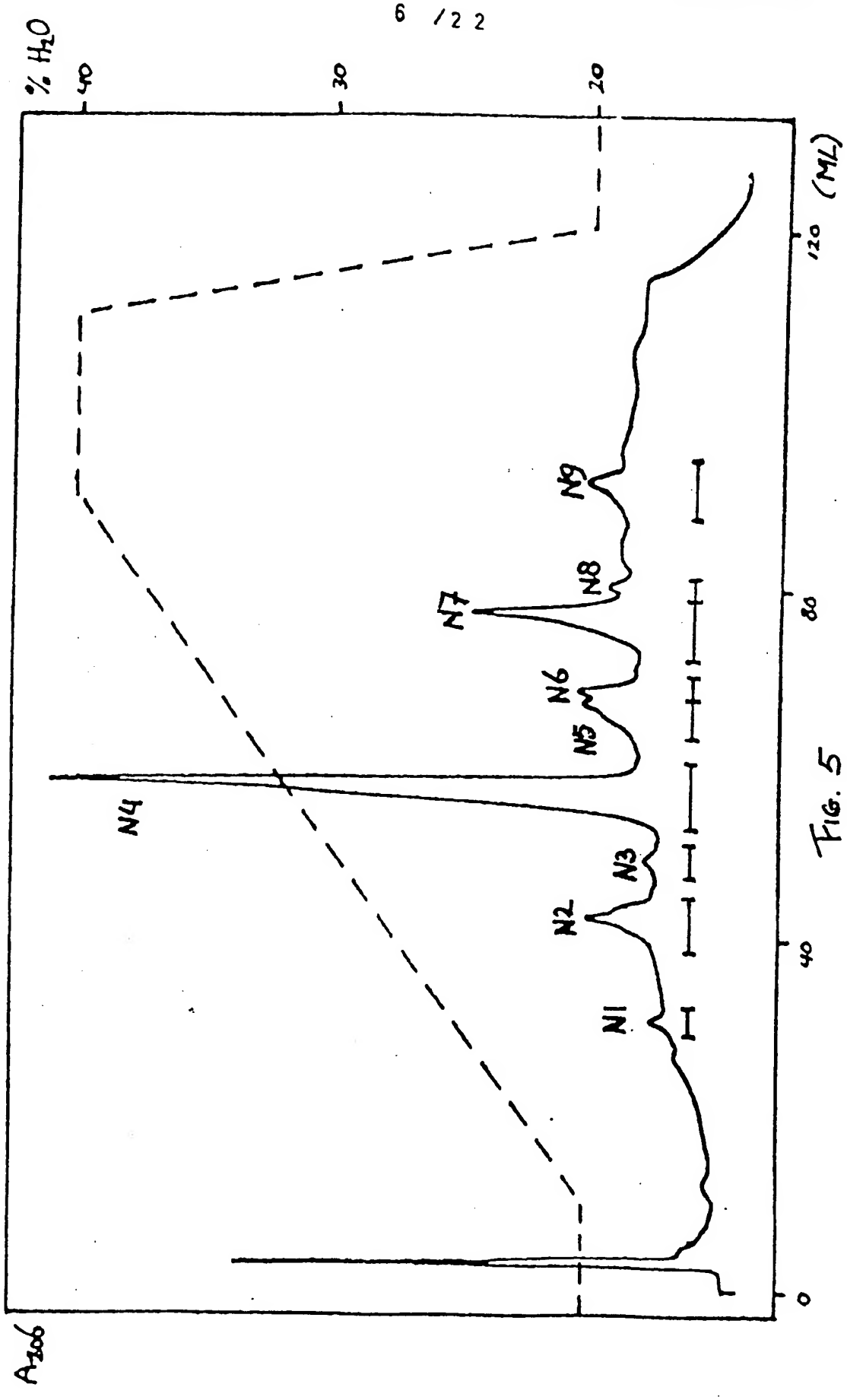


FIG. 5

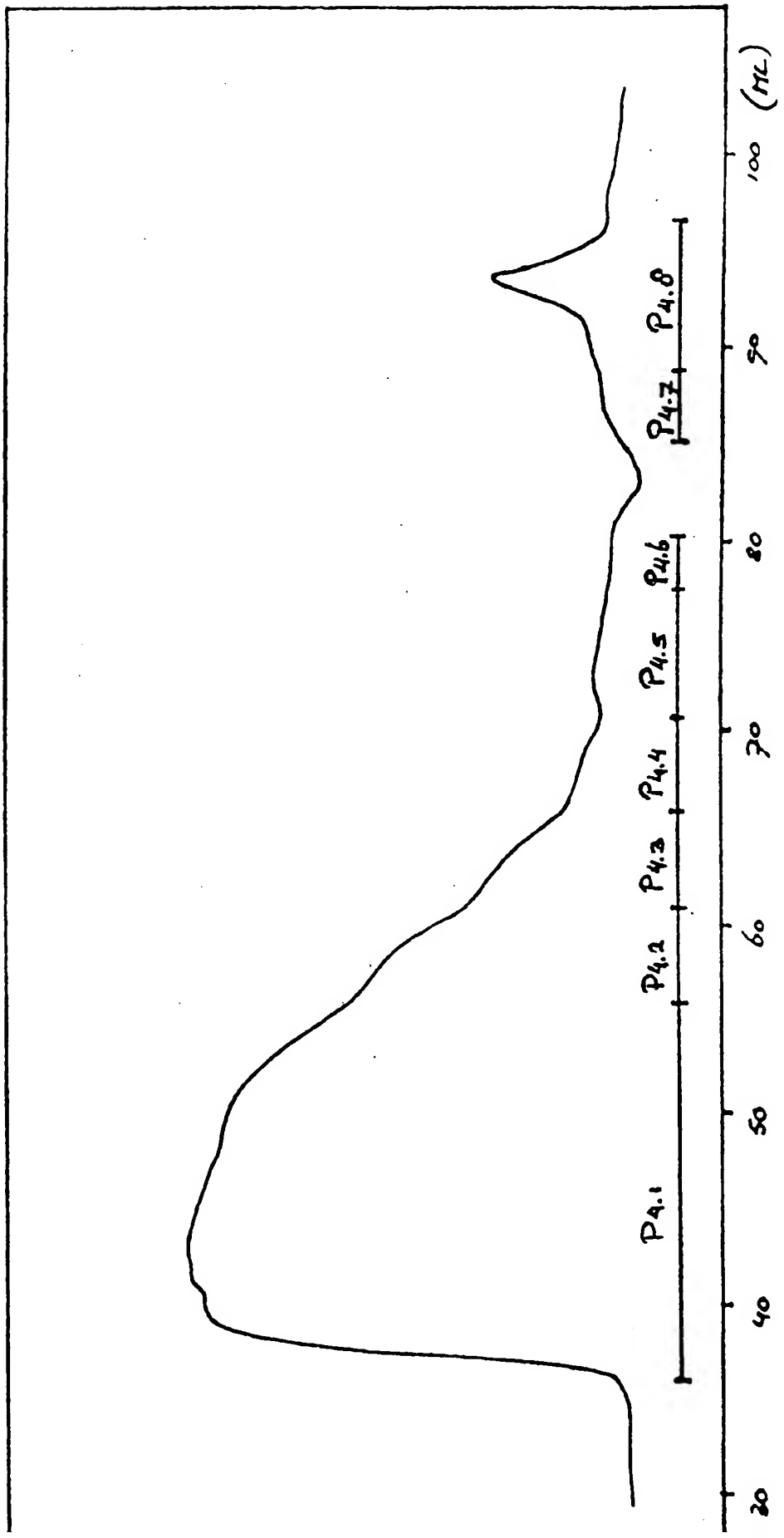
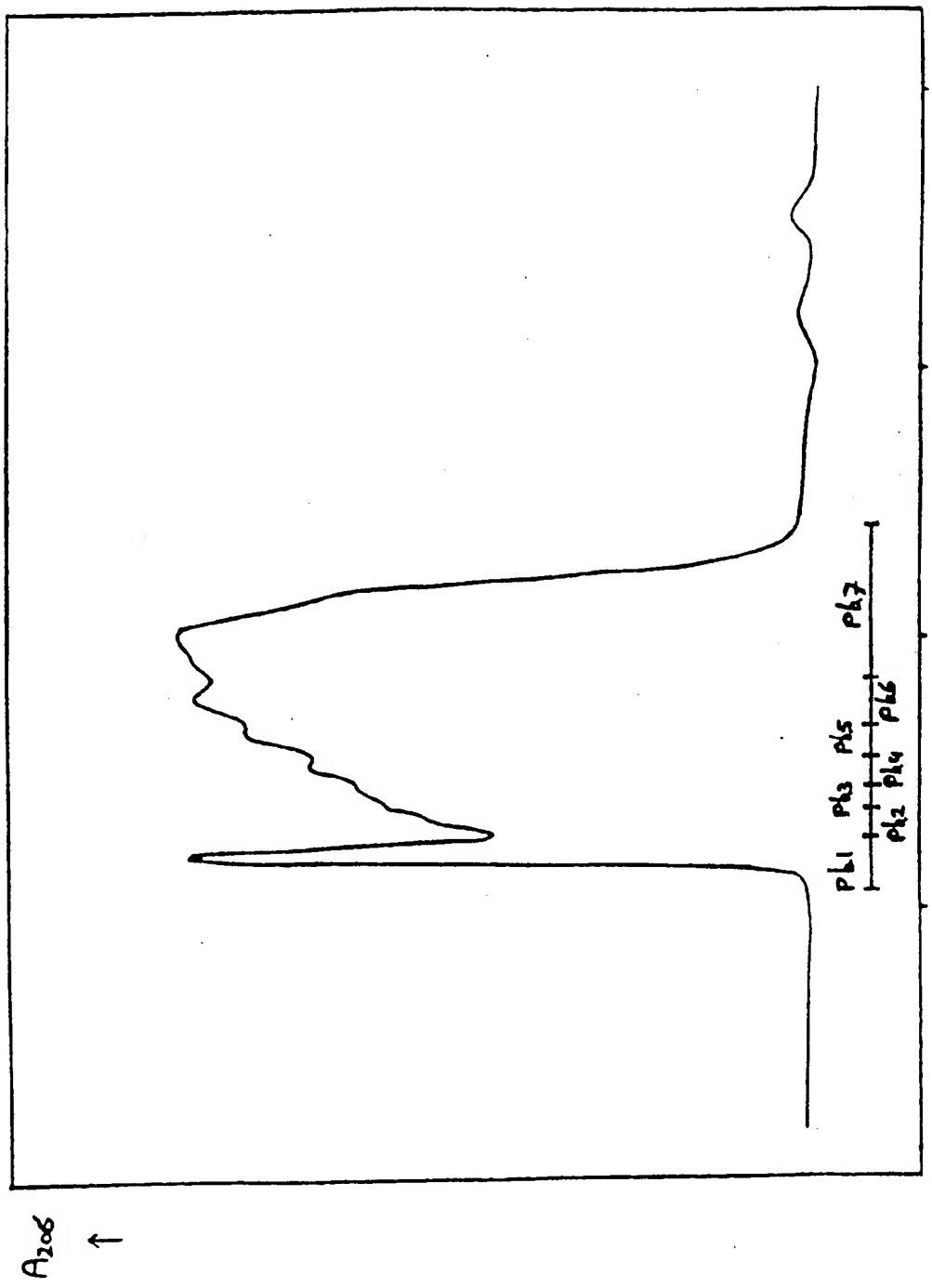


Fig. 6


Pk1
Pk3
Pk5
Pk7
Pk2
Pk4
Pk6
A206

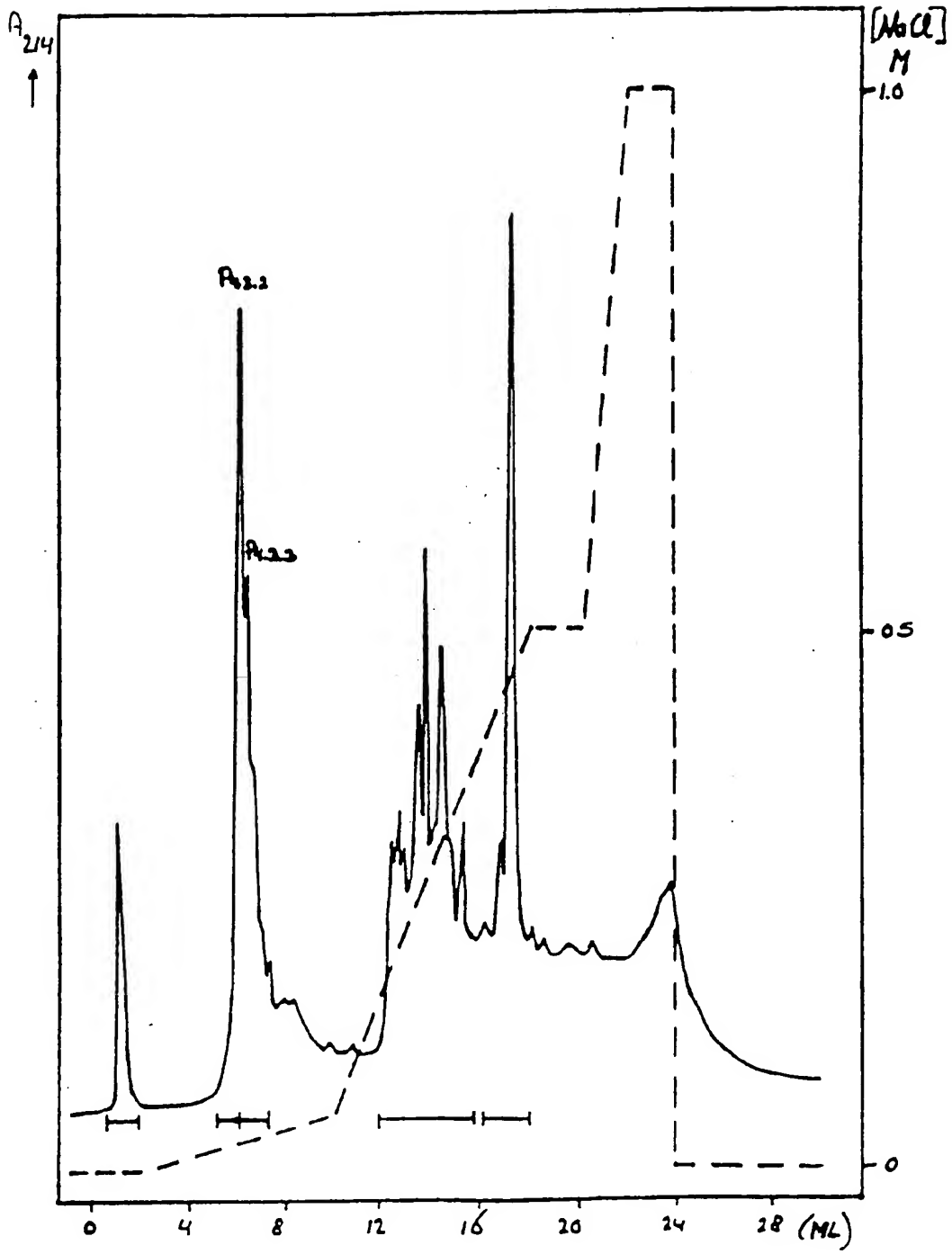


Fig. 8a

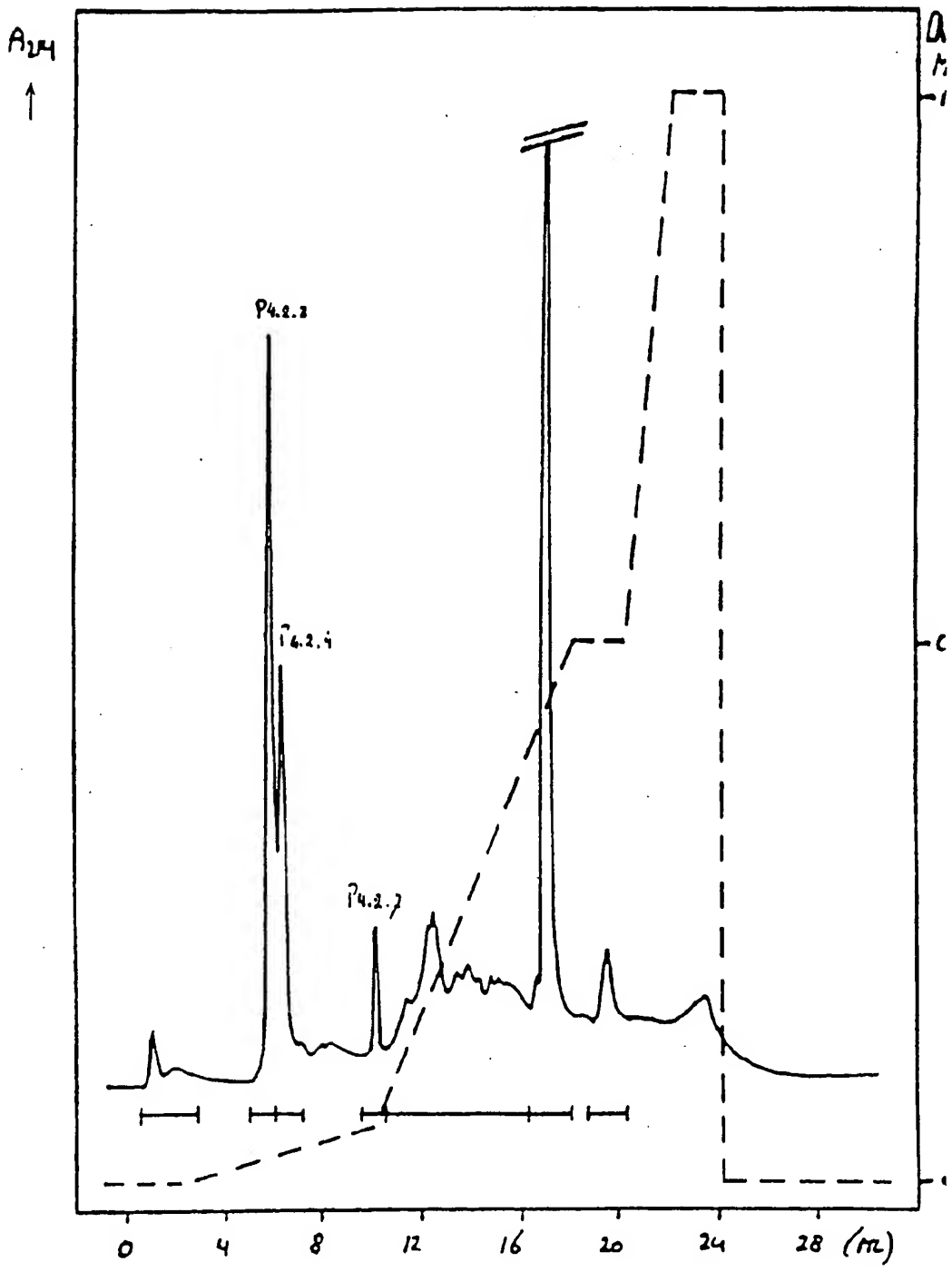


Fig 8B

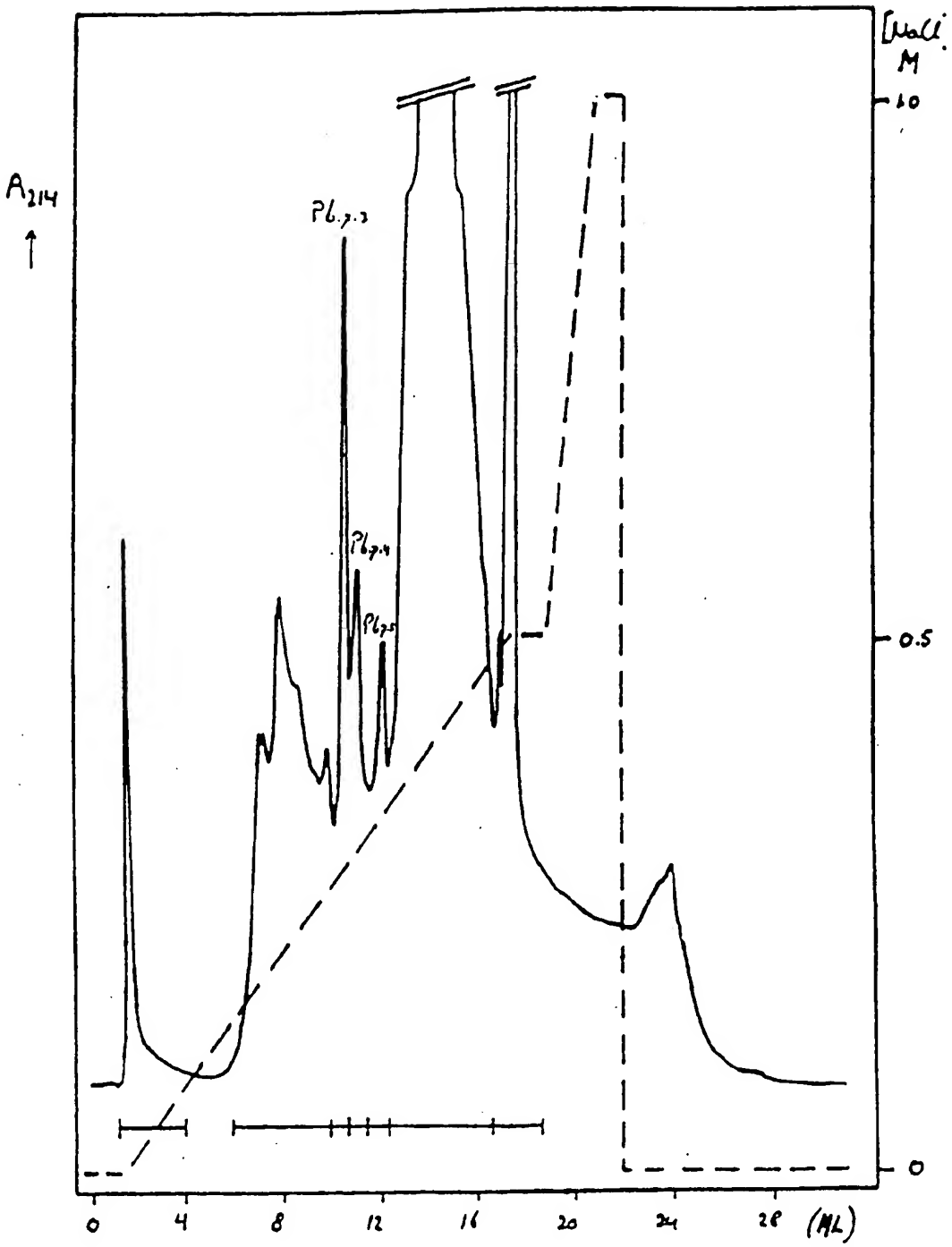


Fig. 8C

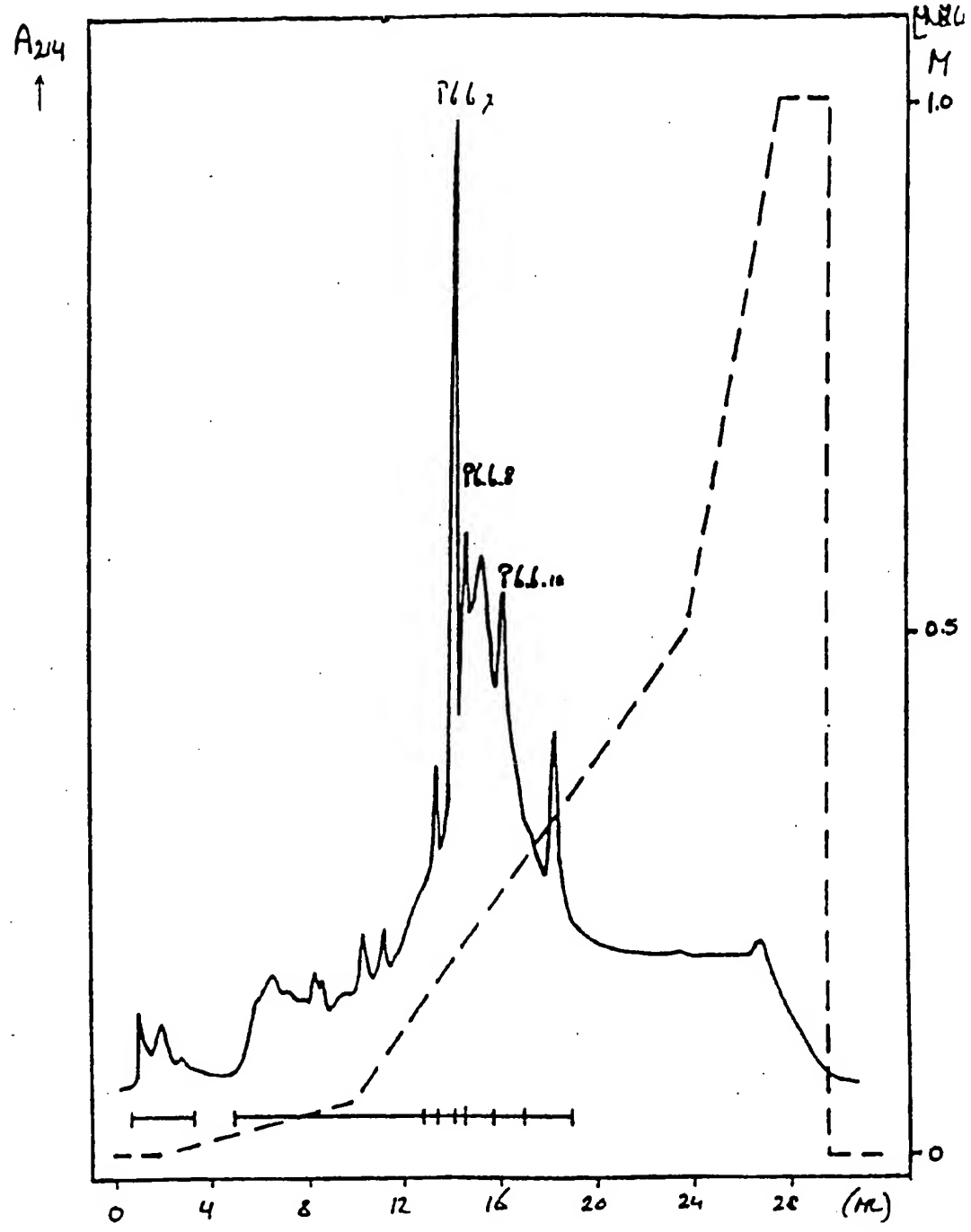


Fig 8D

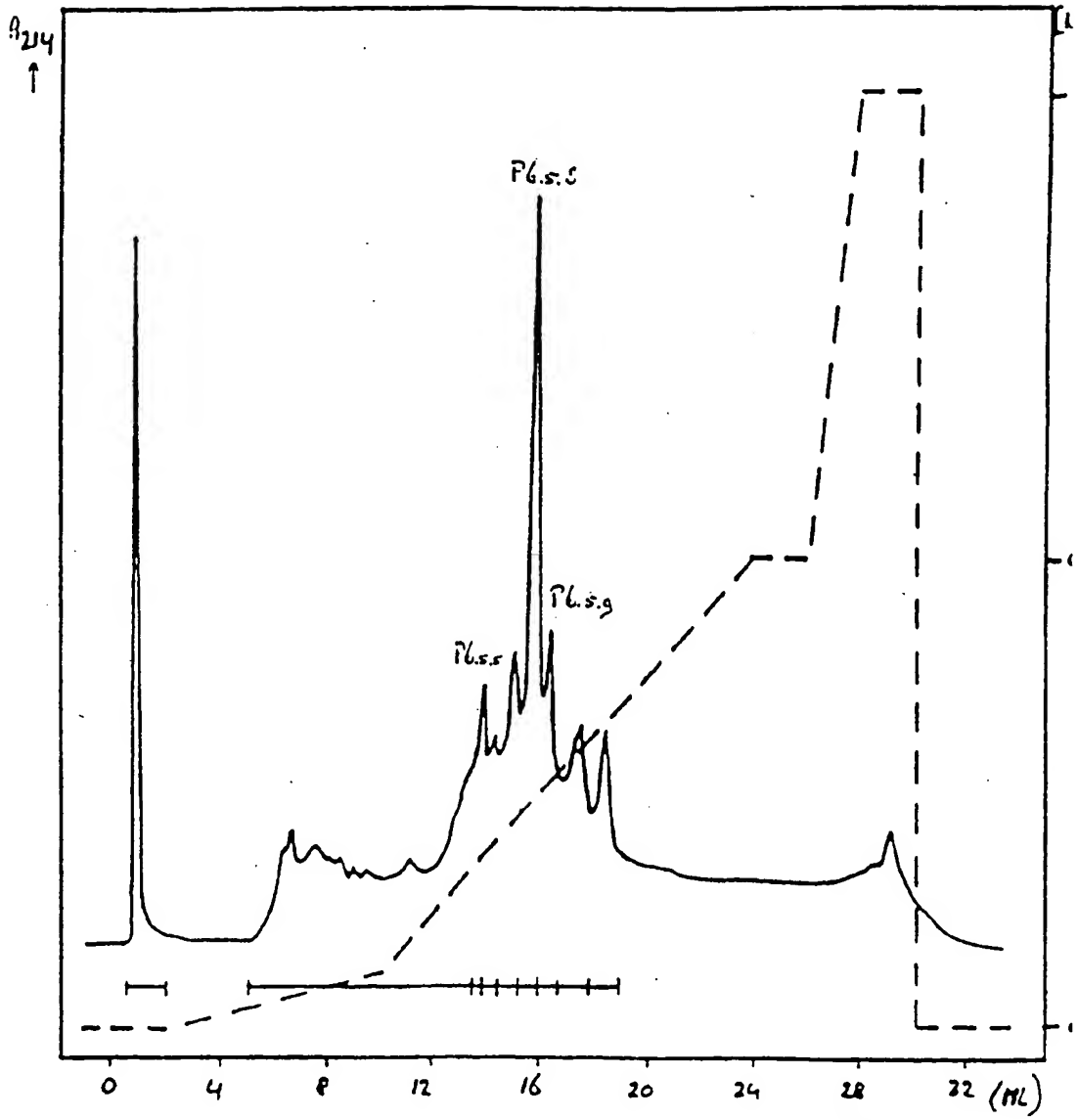


Fig 8E

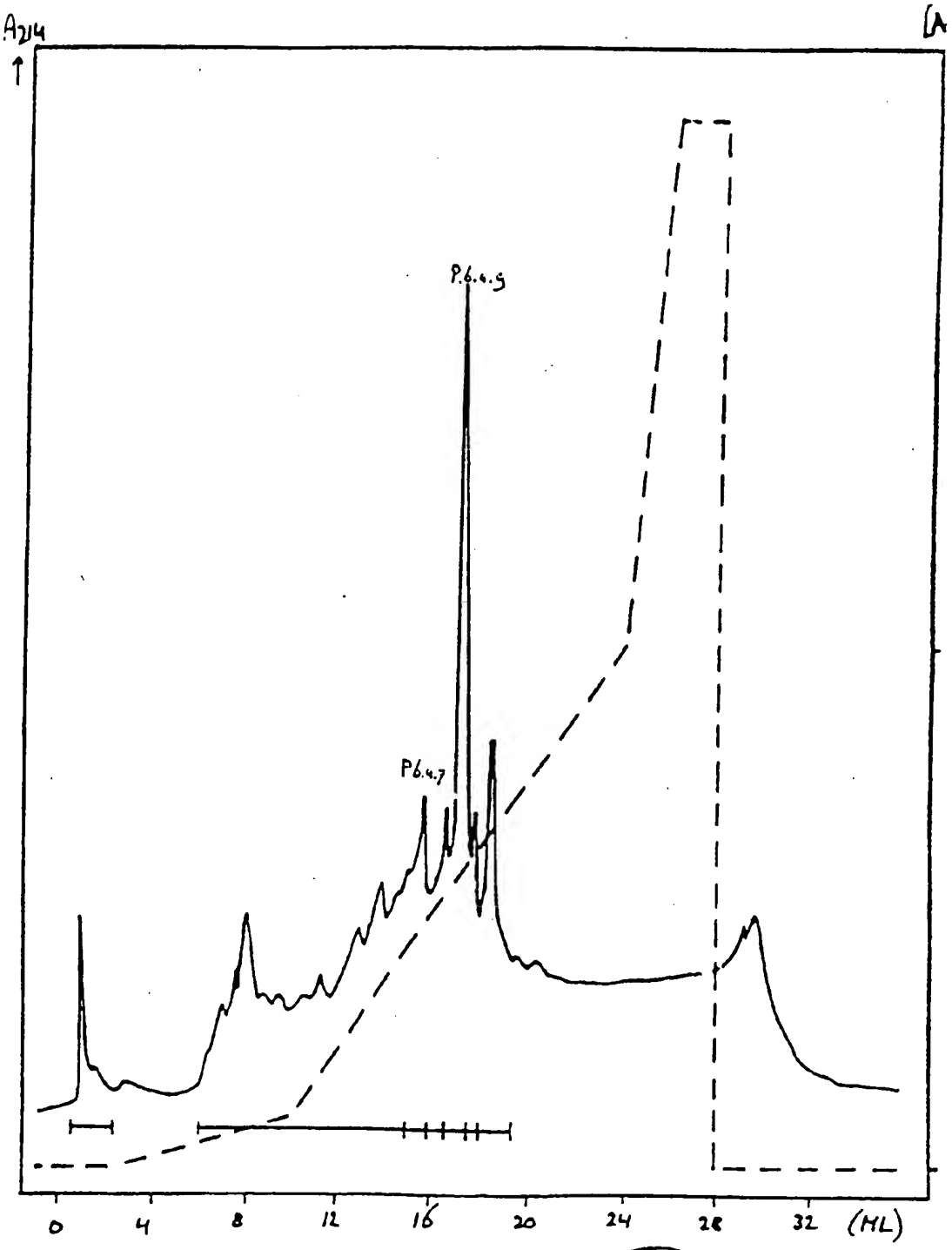


Fig. 8F

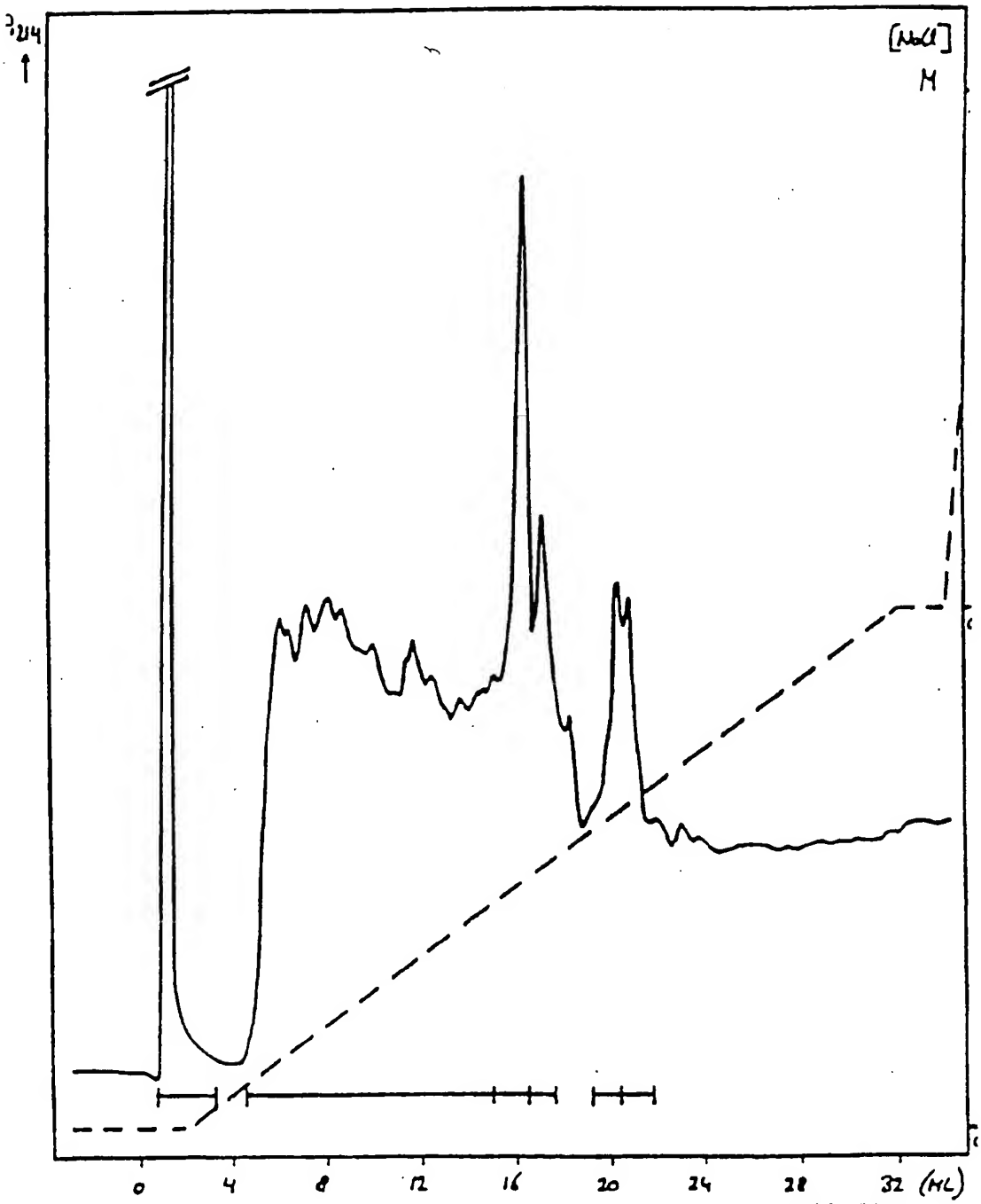


FIG 8G

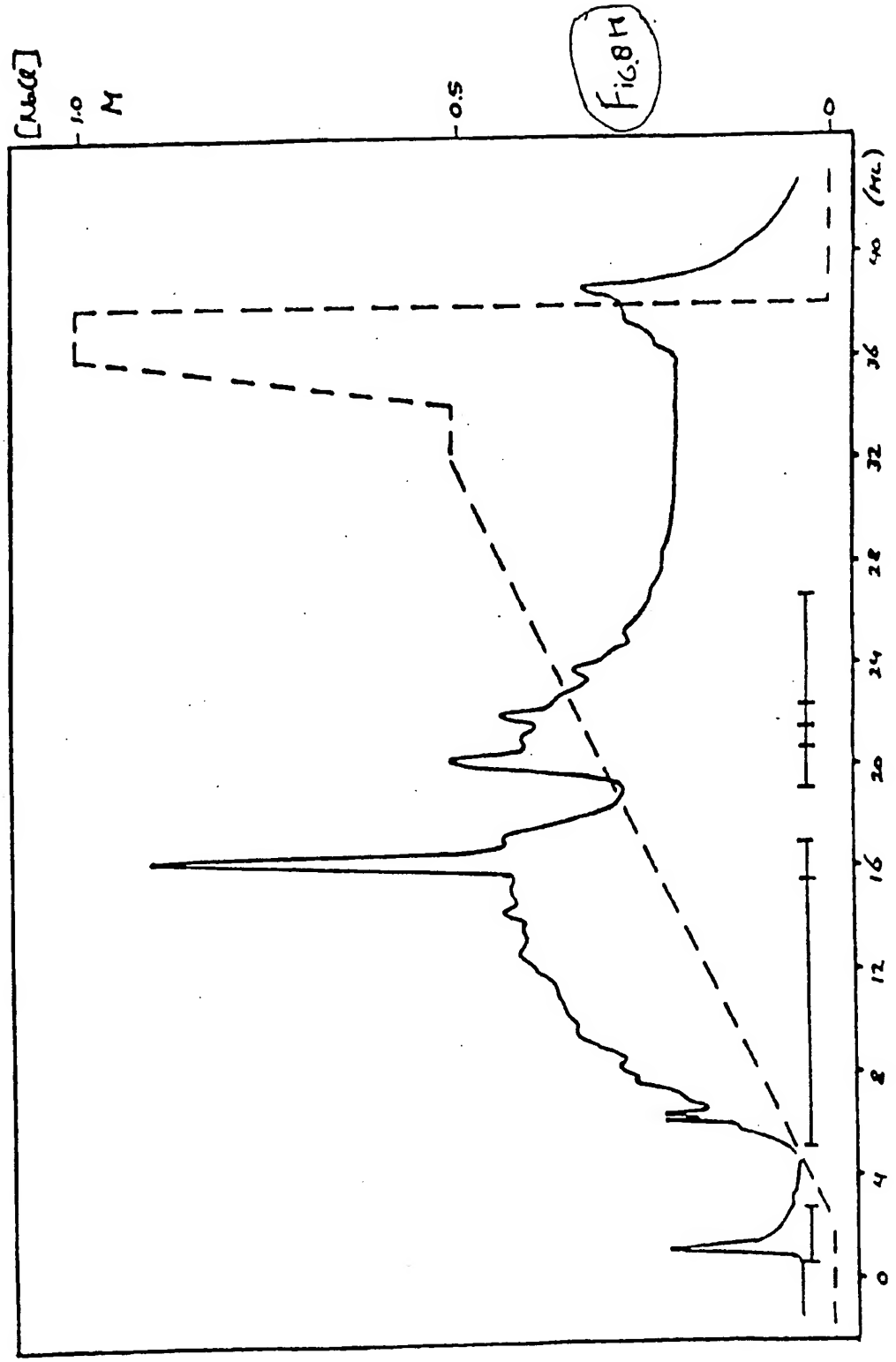

[NaCl]
1.0
M
0.5
0
Fig. 8H
40
36
32
28
24
20
16
12
8
4
0
(mL)

Fig. 9

GENERAL STRUCTURE OF O-LINKED CHAINS

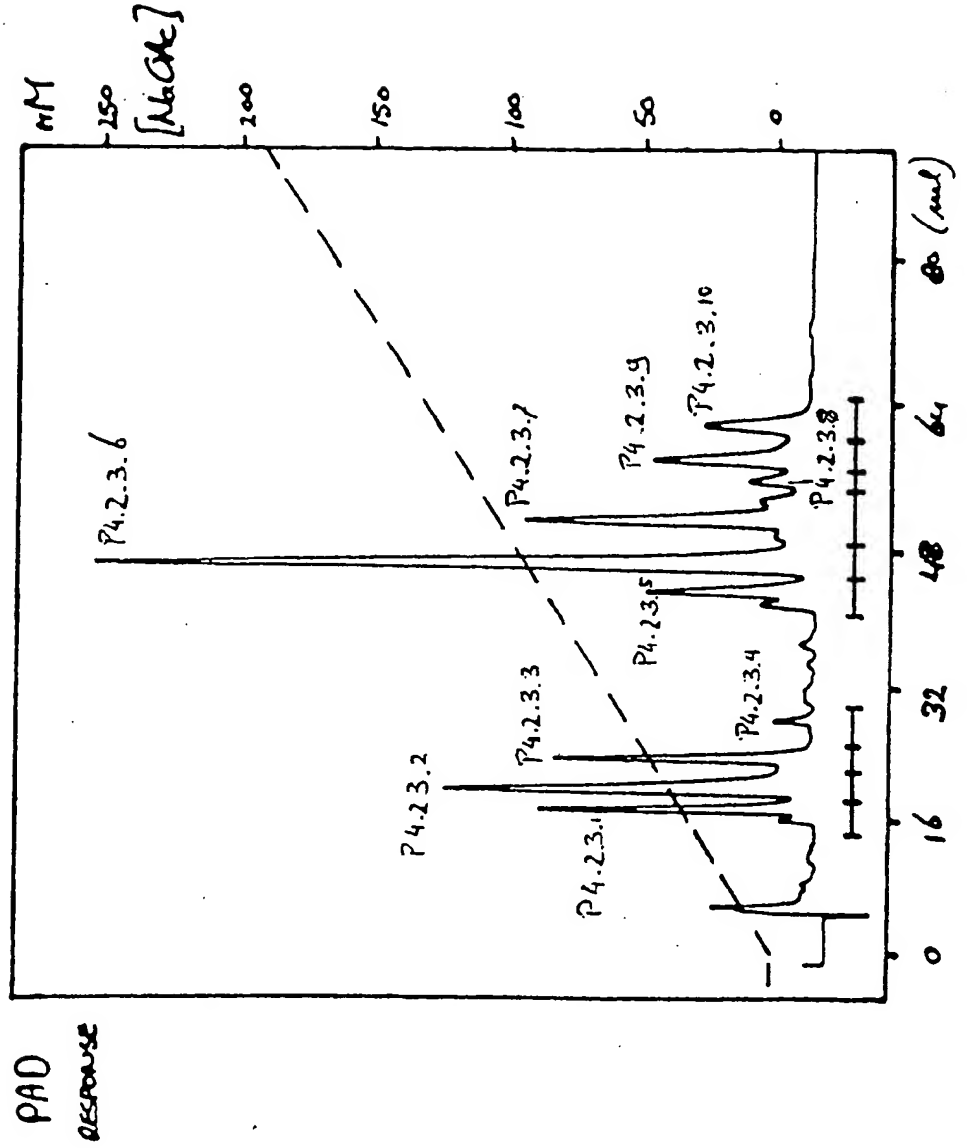

PAD
RESPONSE
nM
[NaOH]
150
100
50
0
0
16
32
48
64
80 (ml)
P4.2.3.6
P4.2.3.7
P4.2.3.9
P4.2.3.10
P4.2.3.8
P4.2.3.5
P4.2.3.4
P4.2.3.3
P4.2.3.2
P4.2.3.1

FIG. 10

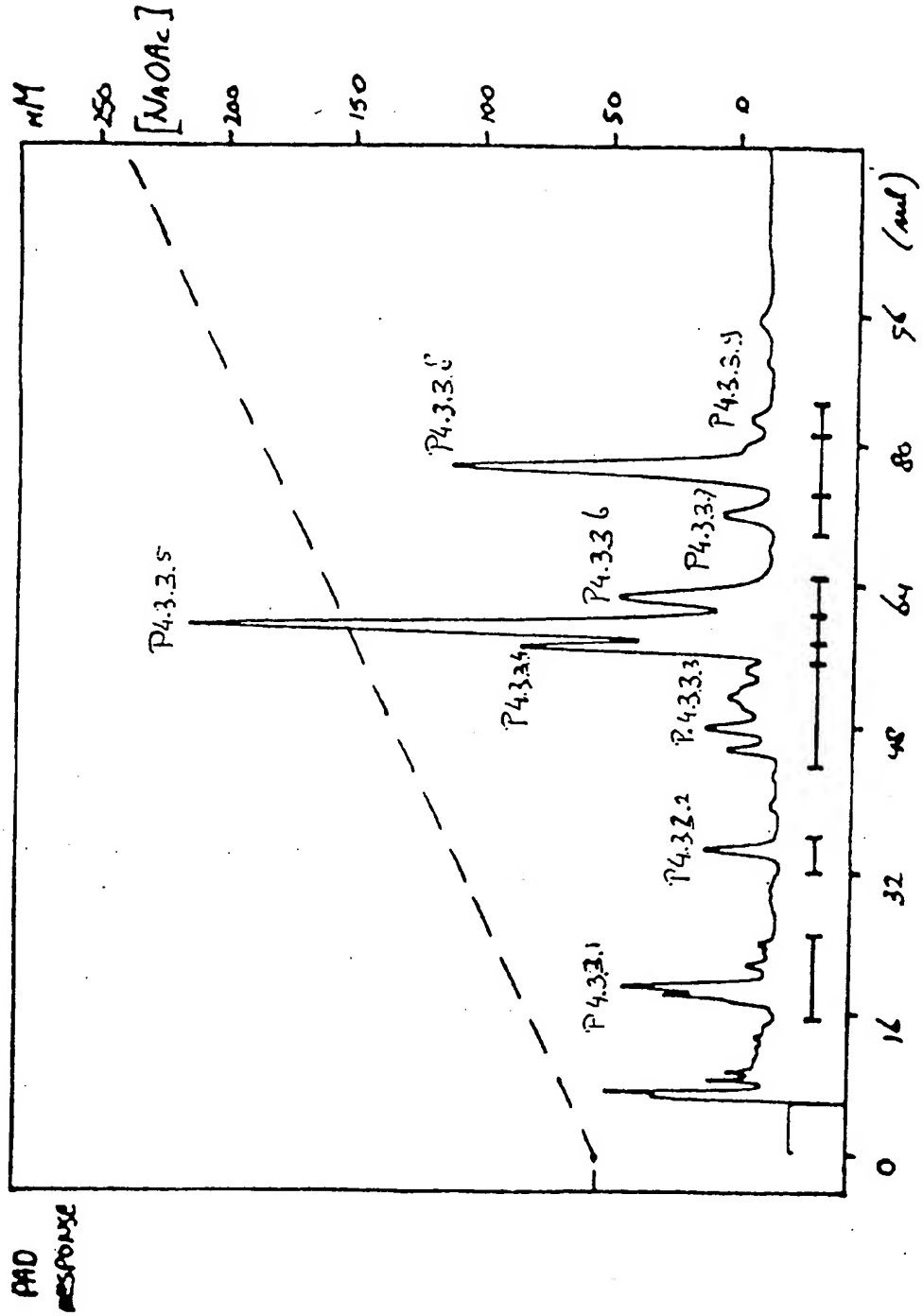

PAD response
mM
[NaOAc]
250
200
150
100
50
0
P4.3.2.1
P4.3.2.2
P4.3.3.3
P4.3.3.4
P4.3.3.5
P4.3.3.6
P4.3.3.7
P4.3.3.8
P4.3.3.9
0
16
32
48
64
80
96
(ml)

FIG. 10

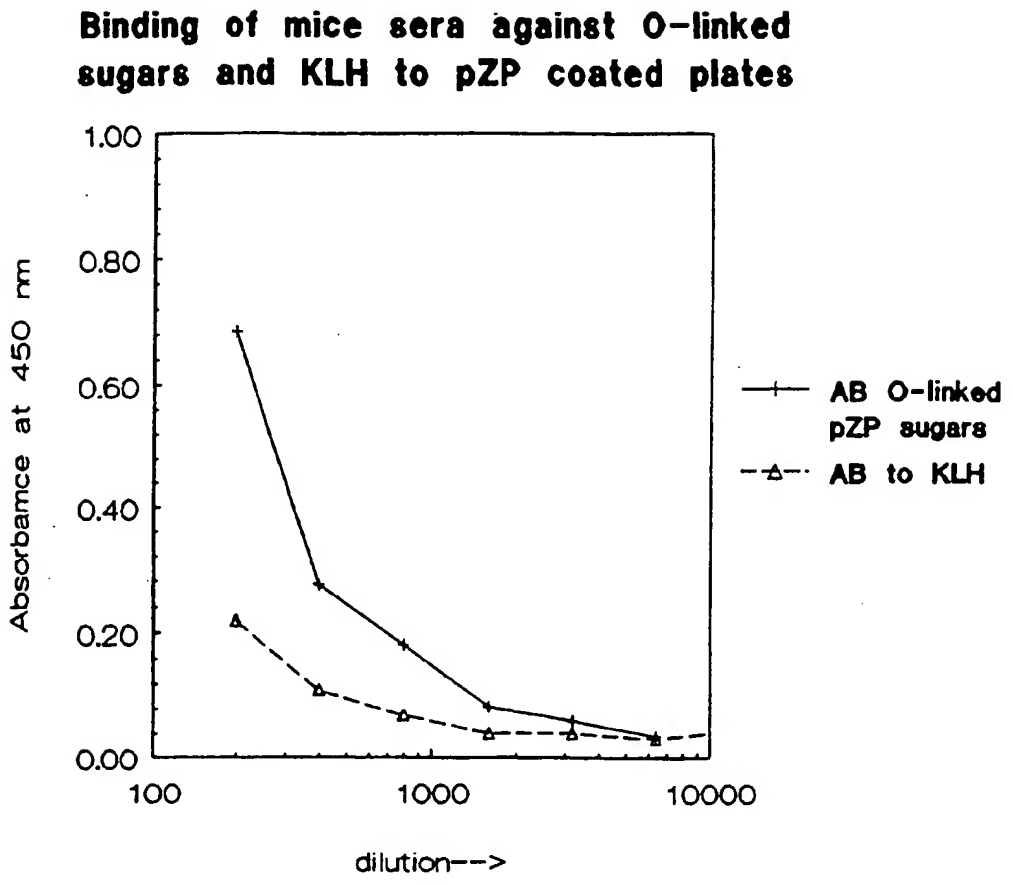

Binding of mice sera against O-linked sugars and KLH to pZP coated plates
1.00
0.80
0.60
0.40
0.20
0.00
Absorbamce at 450 nm
100
1000
10000
dilution-->
AB O-linked pZP sugars
AB to KLH

FIG. 11.

WO 94/19361 PCT/EP94/00569

Effect of AB against O-linked sugars of pZP on porcine sperm binding to ZP

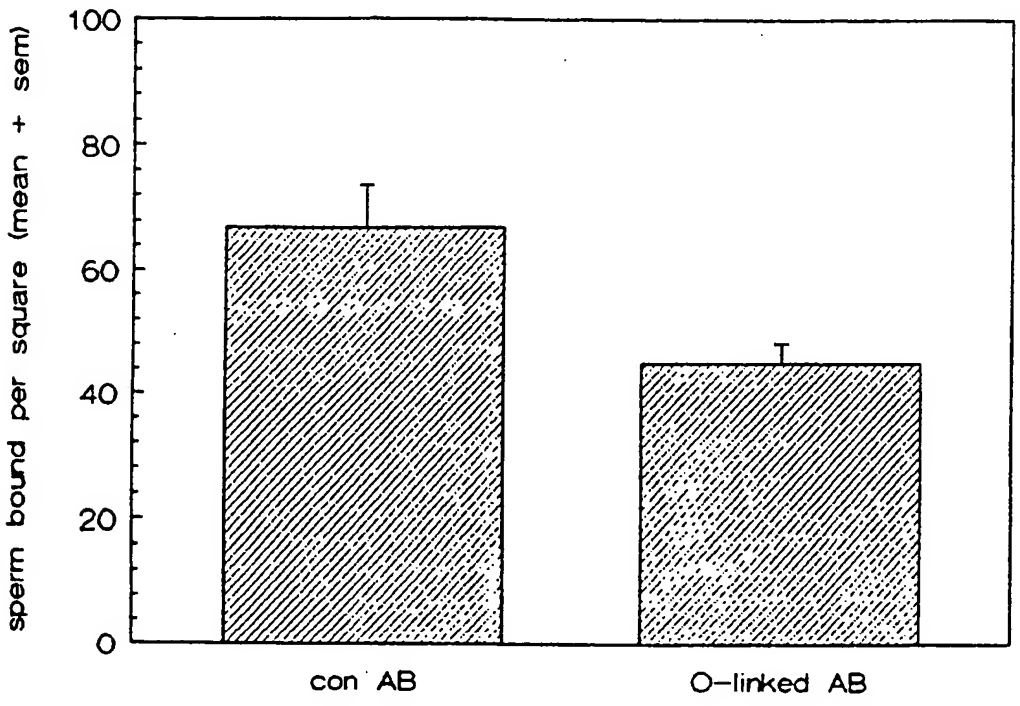


FIG. 12

FIG. 13A EFFECT OF SUGARS ON SPERM BINDING IN HUMAN ZONA BINDING ASSAY HZB9224

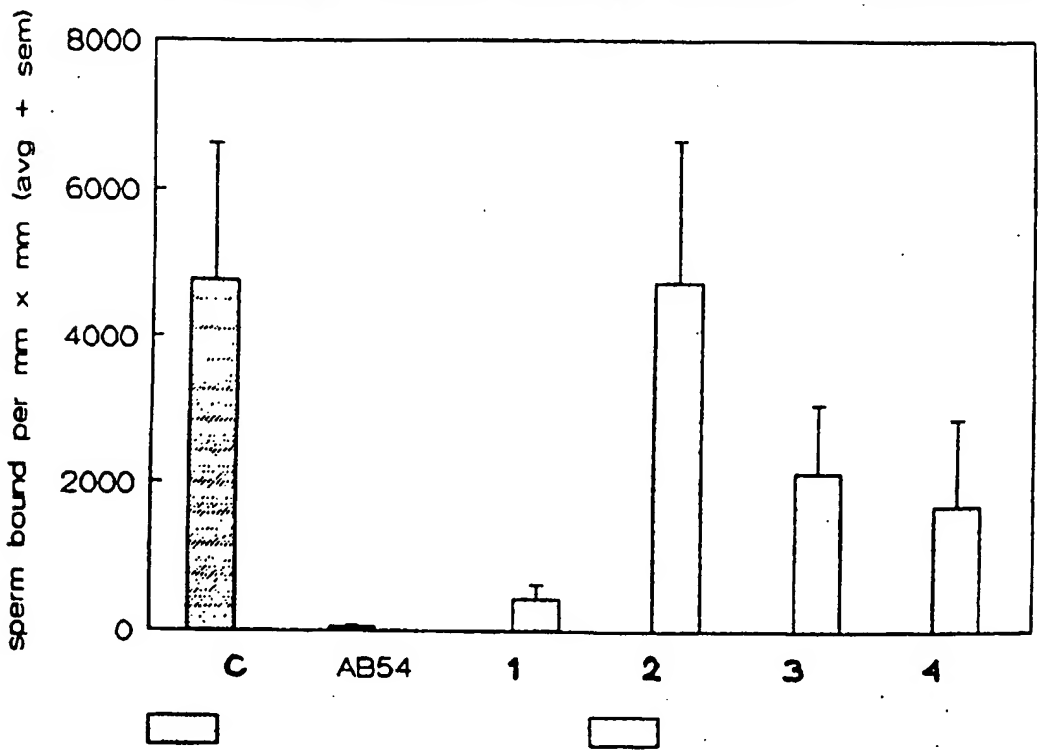
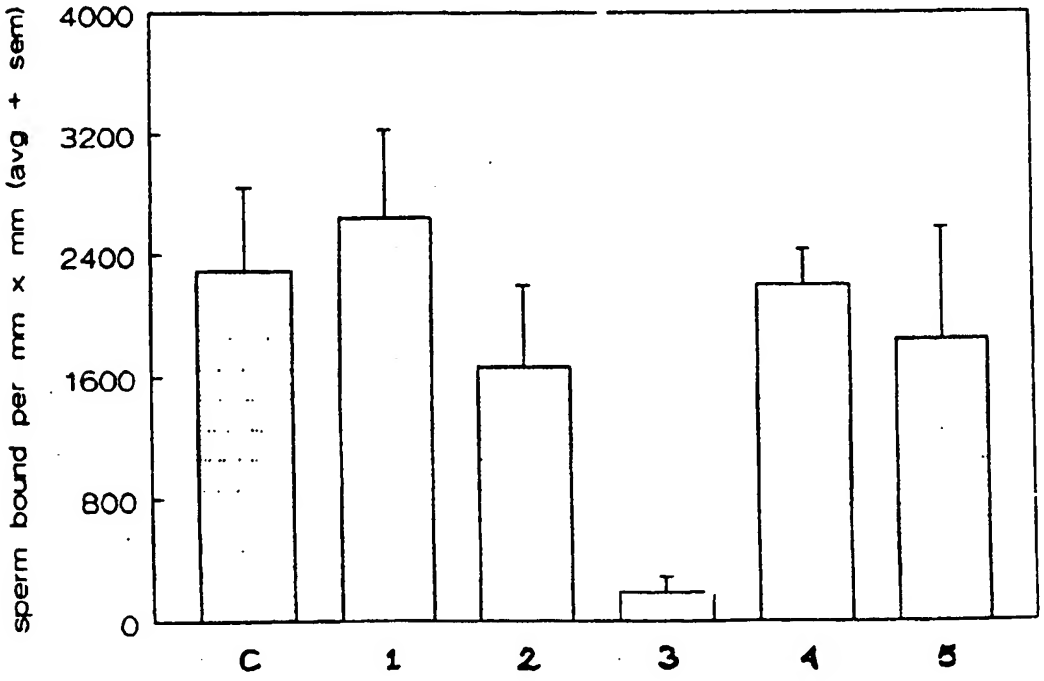


FIG. 13B EFFECT OF SUGARS ON SPERM BINDING IN HUMAN ZONA BINDING ASSAY (HZBA9235)



INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 94/00569

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C07H13/04 C07H15/04 C07H1/08 G01N33/53 C12P21/08
A61K39/00 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07H A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 298 064 (BIOCARB AB) 4 January 1989 see claims; examples ---	1,2
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 85, no. 18 , September 1988 , WASHINGTON DC, US pages 6778 - 6782 J. D. BLEIL ET AL 'Galactose at the nonreducing terminus of o-linked oligosaccharides of mouse egg zona pellucida glycoprotein ZP3 is essential for the glycoprotein's sperm receptor activity.' see the whole document --- -/--	1,2

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
26 May 1994	06.06.94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Moreno, C

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C.(Conunuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIOCHEMISTRY vol. 30, no. 8 , February 1991 , WASHINGTON DC, US pages 2078 - 2087 E. MORI ET AL 'Neutral oligosaccharide structures linked to asparagines of porcine zona pellucida glycoproteins' cited in the application see the whole document	1,2
A	US,A,4 801 689 (T. OIKAWA) 31 January 1989 see column 4, line 9 - line 29	1,2,8
A	CHEMICAL ABSTRACTS, vol. 101, no. 3, 16 July 1984, Columbus, Ohio, US; abstract no. 21934b, 'Antigen-sensitized latex reagent for infertility diagnosis.' page 478 ;column 1 ; see abstract & JP,A,58 216 957 (TOYOBO CO. LTD.) 16 December 1983	10

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		JP-A-	1045313	17-02-89
US-A-4801689	31-01-89	JP-C-	1512915	24-08-89
		JP-A-	62016422	24-01-87
		JP-B-	63061320	28-11-88
		US-A-	4847363	11-07-89
JP-A-58216957	16-12-83	NONE		